

DEFINING GUINEA PIG MONOCYTE HETEROGENEITY  
USING CELLS SEPARATED BY COUNTER-FLOW  
CENTRIFUGATION ELUTRIATION

By

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Dedicated to Sigurd Johns Normann,  
my mentor and friend

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Abstract of Dissertation Presented to the Graduate Council  
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DEFINING GUINEA PIG MONOCYTE HETEROGENEITY USING CELLS  
SEPARATED BY COUNTER-FLOW CENTRIFUGATION ELUTRIATION

By

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August 1983

Chairman: Sigurd J. Normann, M.D., Ph.D.  
Major Department: Pathology

Counter-flow centrifugation elutriation (CCE) separates human blood monocytes into 2 subpopulations that differ in modal volume, Fc receptors and tumoricidal activity. Further studies would be facilitated if an animal model could be found which displayed a similar heterogeneity. The guinea pig was selected for examination because its hematopoietic system is similar to man. Greater than 95% of guinea pig peripheral blood monocytes were recovered by centrifugation over Ficoll-Hypaque (F/H) of specific gravity 1.101. Post-F/H mononuclear cells were injected into a Beckman elutriator chamber at a rotor speed of 3,000 rpm and a loading flow rate of 10.0 ml/min. Cell fractions were collected at 2 predetermined flow rates of 24 ml/min (400 ml collection volume) and 28 ml/min (200 ml

collection volume). Further, the 24 ml/min fraction was collected in two separate aliquots (200 ml each) and designated 24A and 24B. Residual (larger) cells still in the chamber were collected by turning the elutriator rotor-off (R/O) and continuing medium flow. The smaller guinea pig monocytes were found in the 24A fraction and the largest in the R/O fraction. Further functional characterization was performed on the small (24A), intermediate (28 ml/min) and large monocytes (R/O).

Small monocytes had a modal volume (MV) of  $283\mu^3$  and were weakly adherent, non-specific esterase and Fc receptor positive, moderately phagocytic, but negative in acid phosphatase and ADCC activity. This fraction contained the highest level of native tumoricidal activity when tested against the xenogeneic P-815 mastocytoma. Intermediate-sized monocytes (MV  $317\mu^3$ ) demonstrated moderate adherence, non-specific esterase, acid phosphatase, ADCC, and phagocytic activity but were negative for Fc receptors and tumoricidal activity. Large monocytes (MV  $354\mu^3$ ) were strongly adherent, esterase positive, highly phagocytic, Fc receptor positive and active in ADCC. They showed moderate reactivity for acid phosphatase and weak tumoricidal activity.

We concluded that heterogeneity does exist in the guinea pig monocytes and that their reproducible separation by CCE provides a model system with which to further characterize the biology of circulating monocytes.

## INTRODUCTION

A century ago, Eli Metchnikoff introduced the word macrophage to describe those cells in fixed connective tissue which were mononuclear and had the properties of ameboid motion and phagocytosis (136-138). Subsequently, Landau (111) and Aschoff (10-11) noted that non-granulocytic phagocytes were widely distributed in the body based on their staining affinity for vital dyes such as lithium carmine and trypan blue. In 1924, Aschoff proposed that these cells in different body cavities were related not only by morphology and staining characteristics but also by function; he called these cells the reticuloendothelial system (11).

In present day usage, the term mononuclear phagocyte is often used to describe the major cell group originally ascribed to the reticuloendothelial system. The modern concept of mononuclear phagocytes includes cells from all organs and cavities of the body. Fixed tissue macrophages or histiocytes are relatively immobile phagocytes found in connective tissue, bone marrow and all organ systems. In some tissues, they constitute a significant percentage of the total cells. For instance, the Kupffer cells are fixed macrophages constituting one-third of the cells of the

liver (159). In addition, a population of mobile macrophages are widely distributed in the body as exemplified by the circulating monocytes as well as pleural and peritoneal macrophages. Macrophages are a major cell component at sites of inflammation. These macrophages are derived from circulating monocytes (76,216,218) of bone marrow origin (227) and at the inflammatory site they may undergo differentiation to a variety of recognized cell descendants (199,200). In a chronic focus of inflammation, the cells derived from circulating monocytes include macrophages, epithelioid cells and multi-nucleated giant cells (200). Although controversy still exists among investigators, other cell types are strong candidates for inclusion into the mononuclear phagocyte system. One such cell is the Langerhans cell, a sparsely distributed, supra-basal dendritic cell found interdigitating between keratinocytes in the epidermis (66,98). The cells responsible for the remodeling and reutilization of bone, the osteoclasts, have been included also in the mononuclear phagocyte system (12,32,54,157) as well as the microglial cells of the brain and mesangial cells of the kidney (106,175,207). Other investigators include the reticular cells of lymph node and thymus as constituents of this system (68,87,224,225). Indigenous or inducible properties which are shared by all these cell types include adherence, phagocytosis, non-specific esterase activity, the presence of Fc receptors and lysozyme secretion (217).

### Origin of the Mononuclear Phagocytes

It is currently accepted that the blood monocyte is a direct precursor of the macrophage. Van Furth has eloquently demonstrated with in vivo labeling studies that monocytes leave the peripheral circulation and mature into the various macrophages found throughout the tissues (218). Monocytes have relatively long circulation times with half-lives of 17 hours in mouse and 71 hours in man (220). The majority of monocytes do not divide under normal conditions and thus they represent the penultimate form of the mature phagocyte (218, 221). Some investigators believe that a small percentage of monocytes still retain the potential for division after leaving the circulation (215). At present, there is considerable debate as to whether these cells represent a more immature precursor cell, a specific subpopulation of monocytes or derive from a reactivation of otherwise mature monocytes due to factors in the microenvironment.

The earliest cell types which can be clearly identified as precursors of the monocyte are the monoblasts and promonocytes of the bone marrow. In mice, there are estimated to be only  $2.5 \times 10^5$  monoblasts and  $5 \times 10^5$  promonocytes (219,221). Under steady state conditions, the monoblast has a cell cycle time of 11.9 hours and gives rise to two promonocytes (77). Promonocytes have a cell cycle time of 16.2 hours and give rise to two monocytes (219). When inflammatory states alter normal homeostasis,

both monoblast and promonocyte cell cycle times are considerably reduced, leading to an enhanced efflux of monocytes from the bone marrow and into the circulation.

It is difficult to identify the earliest progenitor cells of the macrophage with present-day technology, although indirect assays for their characterization and enumeration have been developed. These progenitor cells are identified by the colonies they form when plated in semi-solid agar and are quantitated in colony forming units (CFU) (76,77). The first step in the generation of the macrophage is thought to be the division of an uncommitted pluripotent stem cell (CFU-S) that gives rise to a stem cell committed to the formation of either granulocytes or macrophages. These colony forming cells are designated GM-CFC. Metcalf believes that two types of progenitor cells exist in bone marrow that give rise to the tissue macrophage pool (134). The first progenitor, GM-CFC, produces mixed colonies of macrophages and granulocytes, granulocytes alone or macrophages alone under the appropriate stimulation by colony stimulating factors (CSF). The second precursor is committed solely to the production of macrophage colonies (M-CFC) (30,114,116,117). The latter progenitor cells are found not only in bone marrow but also in peritoneal exudates, blood and other organs. The significance of these macrophage-colony forming cells (M-CFC) to the total macrophage pool is unclear. However, this self-replicating pool of

macrophages is being investigated to determine if local production of macrophages is a normal event. Volkman argues for the existence of a self-replicating pool of macrophages within the tissues which are not of bone marrow origin (226). This recently described M-CFC could account for this extramedullary source of macrophage precursors.

Two types of CSF have been identified (134). The GM-CSF (syn.CSF II) cause differentiation of the formerly described progenitor cells (GM-CFC) to either pure colonies of macrophages or granulocytes or to mixed colonies as in the majority of cases (20,27,135). Changing the concentration of GM-CSF in the medium will alter the granulocyte/macrophage colony ratio (133). The latter progenitor cell, M-CFC, will differentiate into macrophage colonies under the influence of GM-CSF (116). The second CSF that has been isolated, M-CSF (syn.CSF I), interacts exclusively with the GM-CFC precursors to produce macrophage colonies (201,202).

#### Techniques for Obtaining and Isolating Macrophages

Macrophages are found in association with a myriad of cell types in sundry anatomic locations. Isolating them in high yield and purity is prerequisite to an effective study of their functions and properties. Although a variety of methods have been developed to isolate and purify macrophages, only two of these techniques will be described here.

#### A. Selective Increase in the Macrophage Population

A common method used to obtain workable amounts of macrophages involves their selective induction at a particular site by a variety of stimuli. Intraperitoneal injection of thioglycollate, proteose-peptone, BCG, or Listeria monocytogenes causes an increase in both the absolute number and relative percentage of macrophages within the exudate population (9,34,50,51,57,58). Intraperitoneal injection of Listeria also will cause an increase in the absolute number and relative percentage of monocytes in the peripheral blood without altering other blood cell numbers (5,17). The major disadvantage of such techniques lies in correlating results obtained with such cells to cells obtained under steady state conditions. Such elicited cells may not be indicative of macrophages found under homeostatic conditions. Nevertheless, considerable information on macrophage physiology has been determined using such enrichment techniques. Very commonly, exudates produced in this manner are subjected to additional techniques to purify the macrophages.

#### B. Adherence

One of the earliest and still popular methods for macrophage enrichment is based on the ability of mononuclear phagocytes to adhere to glass or plastic surfaces (33,145,161). Macrophage-enriched populations obtained by inflammatory induction are often the starting

population of cells. This technique was first described by Mosier who noted that a macrophage-rich, adherent population of cells could be obtained after incubation of a mixed leukocyte population on glass plates (145). The cells that were washed off the plates were found to be relatively depleted in macrophages and rich in lymphocytes. These cells were designated as the non-adherent population. Unfortunately, the macrophages had to be used as a monolayer in the same vessel from which they were isolated since they were firmly attached to the glass surface. Later, it was discovered that trypsin or physical scraping of the plate could remove cells for further study (161). However, this resulted in poor recovery and low viability. Subsequently, chelators such as EDTA or the anesthetic, lidocaine, were used to detach adherent macrophages (43,69,168,242). Macrophages were obtained in modest numbers and acceptable viability for subsequent analysis. Nonetheless, this approach was still not ideal as functional damage and a biased selection of macrophages often occurred. Further, cell types other than macrophages reside in the adherent as well as detached population (161). This prompted a search for better methods of purification and recovery.

Newer methodologies take advantage of the ability of macrophages to bind specifically to fibronectin-coated surfaces through interaction with the fibronectin receptor on the macrophage surface (19,167,178). Higher purity of

adherent macrophage populations is possible with this technique compared to glass or plastic surfaces. The adherent cells are easily removed with low concentrations of EDTA as the fibronectin-macrophage interaction is calcium-dependent (1,123). Although cells obtained in this manner are ideal for studying some properties of phagocytes, recovery is neither ideal nor consistent. Furthermore, recent evidence points to the selection of particular groups of phagocytes, leaving the less adherent macrophages in the non-adherent fraction (91,206).

### Techniques for Isolating Monocytes

Monocyte studies have not progressed as rapidly as those using macrophages due to problems associated with recovering monocytes in large enough quantities and sufficient purity for analysis. Monocytes constitute only 2-8% of the white cells in the peripheral blood which also contains vast numbers of erythrocytes and platelets (244). The following methods have been developed to enrich for monocytes and such techniques had to be developed before analysis of this cell type was possible.

#### A. Density Gradient Separation Using Ficoll-Hypaque

The density gradient technique developed by Boyum was a significant achievement that formed the basis of modern methods for isolating the mononuclear cell population from peripheral blood (22). Boyum used density gradients formed

by the high molecular weight polysaccharide polymer, Ficoll, and the radiocontrast preparation, Hypaque. Human peripheral blood, when centrifuged over a 1.079 g/ml density gradient, accumulated lymphocytes and monocytes at the interface between the plasma and the density gradient. Ficoll caused the erythrocytes to stick to each other with the resulting rouleaux formation increasing their specific gravity. Consequently, the erythrocytes sedimented through the gradient and pelleted at the bottom of the tube. The hypertonicity of the Hypaque solution caused granulocyte shrinkage presumably due to the inability of this cell to appropriately regulate its water volume. The increased density and decreased drag force of the granulocytes caused them to be sedimented towards the bottom of the tube. However, the lymphocytes and monocytes were found at the gradient interface. These cells have a lighter density than granulocytes and they are more efficient in controlling their smaller volume of cellular water.

This technique was first developed as a means of isolating lymphocytes from peripheral blood and the monocytes were considered a contaminant. Later, however, this technique was viewed as a method for the recovery of monocytes. By eliminating granulocytes, a 5-10 fold increase was achieved in monocyte purity. Even under the most ideal conditions, however, monocyte concentration is rarely greater than 30% (164,237).

## B. Density Gradient Separation Using Percoll

A more recently developed technique for isolating monocytes from peripheral blood utilizes an isoosmotic suspension of non-toxic polyvinyl-pyrrolidone-coated silica called Percoll. It is used either as a continuous gradient of increasing density (24,79) or as selected densities of Percoll layered successively to form a step gradient (64,208). Isopycnic centrifugation causes cell mixtures of different densities to be separated as each cell type localizes at the specific gravity of the Percoll equivalent to its own density.

It is possible to isolate monocytes in high purity by this technique using either whole blood (64,162) or the mononuclear preparation obtained after Ficoll-Hypaque separation (79). Use of whole blood drastically limits the number and purity of monocytes obtained as the gradients often are overloaded with cells. Mononuclear cell preparations allow the recovery of more monocytes although two separation steps are required with a resultant variable yield.

## C. Fibronectin-Mediated Adherence

Some, but not all monocytes adhere to glass and plastic surfaces (161). Consequently, problems with differential adherence and low yield result. Microexudate and serum-coated plates both contain high levels of fibronectin and have been used to isolate monocytes in

greater than 90% purity and viability (1,67,110,122,123). Again, monocyte recovery is not optimal. Preparative steps often include use of a mononuclear cell preparation obtained by Ficoll-Hypaque separation.

#### D. Counter-Flow Centrifugation Elutriation

The recently developed procedure of counter-flow centrifugation elutriation (CCE) has been used to isolate monocytes (35,62,65,170,187,203,237). Essentially, cells are subjected to centrifugation within the rotor chamber of a continuous flow centrifuge. Centrifugal force, which tends to pellet cells towards the rotor periphery, is counter-balanced by medium flow in the opposite direction towards the rotor center. Under exact predetermined conditions of rotor speed and medium flow, cells remain suspended and reorient within the chamber according to their sedimentation coefficients (118,186). An increase in flow or decrease in rotor speed will cause cells of low sedimentation velocity to leave the chamber while retaining the faster sedimenting larger cells. This results in a clean separation of cells on the basis of size and to a lesser degree, density. There are many advantages in using this technique of separation over the other methodologies described. First, large numbers of mononuclear cells can be introduced into the elutriator for separation (62,65,187,237). Second, most investigators report monocyte recovery and viability in excess of 90%

(62,65,187,237). Third, functional studies conducted on elutriated cells demonstrated no apparent alteration due to the elutriation procedure (62,65,187,237). Lastly, large numbers of monocytes are obtained by a selection method which does not cause major alterations in monocyte physiology due to the selection procedure itself.

### Functions of Macrophages

The macrophage has experienced a renaissance in the last decade with over 50 properties and functions now being ascribed to this particular cell (143). These properties can be grouped into several broad categories which relate to functional roles.

#### A. Antigen Presentation

That macrophages present antigen and help regulate the immune response were recent key discoveries that redefined our concept of immunobiology (209). It is now clear that an interaction between macrophages and T cells is necessary for the normal generation of an immune response. The sequence of events appears to be initiated by macrophage uptake of antigen followed by antigen catabolism that eventuates in some partially degraded material being associated with the macrophage membrane (17,210). This processed antigen becomes closely associated with the I region membrane determinants. Only Ia positive macrophages can participate in presenting antigen and macrophage-T cell

interaction is restricted to cells with histocompatible I regions (18,179,196,209). Specific lymphokines, such as macrophage activating factor (MAF) and macrophage Ia recruiting factor (MIRF), have the ability to increase the number of macrophages possessing Ia and antigen presenting ability (210), while the prostaglandin PGE<sub>2</sub> reduces the number of Ia positive macrophages with resultant down-regulation of the immune response (210).

#### B. Secretory Functions of Macrophages

Macrophages secrete an incredible array of enzymes, plasma proteins and biologic modifiers (143). The bacterial compound, lysozyme, is considered a constitutive product of all macrophages (75). Further, macrophages synthesize the first five components of the complement system (40,143,158). The secretion of neutral proteanases such as plasminogen activator, elastase and collagenase by macrophages is important to the role these tissue cells may play in inflammation (39,183). In addition, major biologic modifiers such as the prostaglandins (25,90), interferon (120,121) and colony stimulating factor (31,72,142) are produced by macrophages.

#### C. Anti-Microbial Functions of Macrophages

Macrophages have the ability to destroy a wide spectrum of procaryotic and eucaryotic parasites (127). Killing usually takes place after fusion of the phagosome

with primary and secondary lysosomes (127). It is speculated that several pathways exist for intracellular microbial killing which may vary depending upon the type of organism and the type of macrophage. A hexose monophosphate shunt (HMS) dependent oxygen burst precedes most intracellular killing events (105,127,146,243). A variety of oxygen metabolites are produced in this manner. Superoxide anion, hydrogen peroxide and other oxygen intermediates are major products generated in phagolysosomes and are thought to be the principal microbicidal agents (56,238). Mechanisms other than those involving oxygen may also function in microbicidal activity. Lysozyme, cationic proteins, acid hydrolases and other lysosomal constituents have all been postulated to play an anti-microbial role. Under certain conditions, microbicidal activity within macrophages is increased and such macrophages are referred to as activated for microbial killing (97). A variety of lymphocyte-derived factors from immune-mediated reactions to antigens (26) and obligate intracellular parasites have the ability to activate the macrophage (155). Although the events leading to activation are not well understood, it is well established that the result is a cell with both heightened oxygen metabolic machinery and microbicidal capacity (127).

#### D. Anti-Tumor Functions of Macrophages

It was a fortuitous discovery that macrophages can kill tumor cells, for the original objective of these studies was concerned with the enhanced ability of activated macrophages to destroy microbes. However, such fully activated macrophages were found capable of destroying tumor cells and virus-infected neoplastic cell lines in vitro and in vivo. Astonishingly, normal cells present in the same culture vessel were spared from attack (83).

Most of the early data on macrophage tumoricidal activity was elucidated by Hibbs and by Keller. Hibbs first demonstrated an increased resistance to both autochthonous and transplantable neoplasms in mice chronically infected with obligate, intracellular parasites (84). Activated macrophages from chronically infected animals were able to destroy tumor cells but not normal cells in vitro. At the same time, Keller had shown that activated macrophages could suppress tumor growth in vivo (101). He demonstrated that adoptive transfer of irradiated peritoneal exudate cells which were functionally composed of activated macrophages were able to suppress tumor growth in total body irradiated syngeneic recipients. Keller later contended that the major effect of activated macrophages was on the proliferative capacity of the tumor and that cytostasis was of varying degrees depending on the target cell tested (103). Both Hibbs and

Keller concluded independently that tumor cell killing by activated macrophages was by a non-phagocytic, non-specific means involving macrophage/tumor cell contact (85,102).

Data from the laboratory of Fidler support this hypothesis in as much as activated macrophages destroyed B16 melanoma cells in culture, but again, did not kill normal cells (59). He then confirmed this observation in vivo by demonstrating that IV injection of activated macrophages into B16 melanoma-bearing mice significantly reduced the number of established pulmonary metastases. Tumor growth was also inhibited at primary sites of inoculation by activated macrophages (45). Furthermore, macrophages from primary sites of immunogenic tumors were tumoricidal in vitro (122,184).

The ability of activated macrophages to recognize and destroy tumor cells has been postulated to depend upon a specific recognition event (60). However, species-specific antigens, tumor-specific or associated antigens, and histocompatibility antigens are not responsible for the selective destruction of neoplastic cells, although macrophage-tumor cell contact has been shown to be necessary for tumor cytolysis to occur (61). Some investigators have demonstrated highly specific binding of activated macrophages to tumor cells (124,125). Such macrophage/tumor cell aggregates can be disrupted by the addition of excess unlabeled target cells. Addition of partially purified tumor cell membranes also suppresses

tumor binding and cytolysis. Normal unstimulated macrophages do not form macrophage-tumor cell aggregates and are not tumoricidal. At present, a specific tumor binding receptor has not been identified.

The mechanisms involved in the actual cytolytic event are poorly understood. Substances such as oxygen intermediates, arginase, and prostaglandins (all of which are made by macrophages) can damage tumor cells (2,4,5). However, high levels of these products seem necessary to affect tumor killing. For example, the amount of  $H_2O_2$  necessary to effect killing of some strains of tumor cells in vitro is far in excess of that which would be synthesized by a quantity of macrophages causing comparable damage (147). A recently discovered neutral proteinase, which is excreted exclusively by activated macrophages and is inhibited by serine proteinase inhibitors, kills tumor cells in low concentrations, e.g., the  $LD_{50}$  for tumor targets is  $1 \times 10^{-9}$  M (4). It is postulated that the tight adherence of macrophages to tumor targets provides a channel for protecting neutral proteinase from the inhibitory effects of plasma. Neutral proteinase has also been found to act synergistically with other macrophage secretory products such as hydrogen peroxide in affecting tumor cell lysis (3).

Another mechanism by which macrophages may bind and kill tumor cells involves specific anti-tumor antibody and interaction with the Fc receptors on macrophages.

Antibody-dependent cellular cytotoxicity (ADCC) is a well known property of macrophages (193,197,233). Originally assayed by measuring radiolabeled chromium release from antibody sensitized erythrocyte targets (141), ADCC has been shown to be a mechanism of lysing nucleated cells as well (109). Essentially all macrophages can participate in ADCC by virtue of their Fc receptors. Direct correlation with expression of Fc receptors and cytolysis has been demonstrated (92,108). Activated macrophages are more effective in mediating ADCC than resting macrophages (107). The mechanism involved in the actual cytolysis of tumor cells is again unclear at this time, but essentially all mechanisms described previously for activated macrophages have been implicated.

Hibbs et al. proposed that macrophages may function in tumor surveillance. He found that activated macrophages were considerably more cytopathic for transformed cell lines in vitro than for normal cell lines which exhibited contact inhibition, anchorage dependence, etc. (86). His work supported the contention that macrophages were a part of a primitive non-immunologic surveillance mechanism capable of detecting and destroying cells manifesting aberrant growth, thus preventing cancer development.

The activated macrophage may not be the only cell capable of immunologic non-specific tumoricidal activity, however. Current work focuses on destruction of nascent tumors by a class of circulating cells called natural

killer (NK) cells. By broad definition, these cells have in common the ability to destroy tumor cells of hemato-poietic origin, across species barriers (80,82,241).

Current data do not unequivocally link NK activity to one particular cell. Herberman has isolated a large granulated lymphocyte (LGL) in high purity from human and murine blood by isolation on Percoll gradients (80). This cell has the properties of a T lymphocyte (surface markers, theta antigen, morphology). Curiously, other investigators have determined that the NK cell is a promonocyte possessing macrophage surface markers but lacking the typical characteristics of the macrophage (phagocytosis, adherence, esterase) (119). These LGL's and promonocytes are capable of destroying tumor cells of hematopoietic origin in accordance with the definition of NK cell activity.

Augmentation of NK activity results in vivo from the addition of immune modulators such as interferon (46,70), poly I:C (153,154), or BCG (163). Infection with viral agents also raises NK activity (47). Eremin has implicated the Kurloff cell of the guinea pig as responsible for NK activity (52). This cell, with its large eosinophilic, eccentrically located inclusion body, has both properties of lymphocytes and macrophages, thus making its origin uncertain (104,171). The techniques used to establish the NK cell activity of Kurloff cells were not definitive, relying upon negative depletion techniques to show loss of NK activity (52).

Macrophages and their precursors have also been implicated as possessing an immune surveillance role. Unlike NK activity which is manifested against a rather restricted tumor target range, the native tumoricidal activity of mononuclear phagocytes covers a broad range of tumor targets, many not of hematopoietic origin (94). Native tumoricidal activity is usually measured over a longer assay time (18-72 hours) (131,205) than NK activity (4 hours) (82). Again, augmentation of tumoricidal activity occurs with the addition of immune modulators (132).

Accumulating data on NK activity and native tumoricidal activity have led some investigators to speculate that more than one cell type is responsible in vivo for affording protection against neoplastic cell growth (37). Mice whose immune systems have been destroyed by high dose irradiation or nude mice lacking a functional T cell system manifest the same rates of spontaneous hematopoietic cancers as normal animals with functionally intact immune systems. Interestingly, no disease states have been described wherein the host lacks a mononuclear phagocyte system. This condition might be incompatible with life for a variety of reasons, including protection against the aberrant growth of neoplastic cells. Therefore, cells of both macrophage and lymphocyte origin may play strategic roles in immune surveillance.

### E. Role of Macrophages in Inflammation and Repair

That macrophages phagocytize foreign matter has been known since the first observations of Metchnikoff (136-138). Thus, it comes as no surprise that macrophages play a crucial role in the inflammatory process.

The temporal events of cell migration and accumulation at an inflammatory site are well established. After the initial wave of neutrophils, monocytes become the principal cell entering the lesion (228). Monocytes are a chemotactically responsive cell and in vitro migrate directionally in response to gradients of C5a or f-met-leu-phe (188,198). In vivo at the site of inflammation, they first adhere to post-capillary venular endothelium, then emigrate between endothelial cells. Once present, macrophages phagocytize dead tissue, senescent granulocytes and particulate debris. It is believed that monocyte secretion of plasminogen activator may be involved in a localized lysis of the basement membrane allowing the monocyte to enter the area of injury (240). Indeed, macrophages are considered the principal cell responsible for wound debridement and for normal wound repair. They appear to secrete a factor(s) involved in fibroblast migration and collagen synthesis (228) as well as neovascularization (165,166). Macrophages in various states of maturation and differentiation are found at sites of inflammation. The chronicity of the lesion, the composition or antigenicity of the phlogistic agent and the immune status of the host determine the

predominant types of mononuclear phagocytes that reside at the inflammatory site.

Once the monocytes have arrived at the site of inflammation, an impressive array of biological interactions and biochemical events takes place that lead to the transformation of monocytes into macrophages. In immune-mediated inflammation an increased modulation of macrophage functions may occur via lymphokine production by T cells (42). Alternatively, immune complexes alone can stimulate macrophage functional activity (41). Phagocytosis seems to be the event that triggers macrophages to secrete a variety of enzymes into the surrounding area (190). Once initiated, secretion becomes a function divorced from the initial phagocytic trigger. The three major groups of enzymes are lysozyme, lysosomal hydrolases and neutral proteinases. Receptor complexes such as C3b-C3b, cause the selective secretion of acid hydrolases (191) whereas C5a stimulates both acid hydrolase and neutral proteinase secretion (129,211). The latter group of enzymes together with reactive oxygen intermediates (56,238) is now thought to be responsible for most of the tissue damage present at sites of chronic inflammation (13). Indeed, macrophages play a key role in perpetuating and actually accentuating the inflammatory process in some disease states (44). Alternatively, the production of prostaglandins by macrophages at inflammatory sites could down-regulate the

local immune response thus leading to a more controlled inflammatory process (14,21,73).

Very few products secreted by macrophages have been analyzed in relation to their role in inflammatory physiology. Nevertheless, considerable progress has been made in understanding the complex biochemical events modulated by macrophages in inflammation, a cell that two decades ago was thought to have the singular function of removing foreign matter and necrotic debris from a nidus of inflammation.

#### Macrophage Heterogeneity

Early work on macrophage function concentrated on characteristics expressed by the cell population as a whole. In analyzing some of the classical properties which characterize elicited macrophage populations, it emerged that considerable heterogeneity exists at the individual cell level with some macrophages even lacking properties of adherence (113,204), non-specific esterase (63), high phagocytic rate (100,181,229), Fc receptors (99,230) and ADCC activity (194). Additionally, considerable differences exist among peritoneal macrophages elicited by various means (74,148,189). Major differences also exist among unfractionated macrophage populations obtained from different sites. For instance, alveolar macrophages rely primarily upon oxidative metabolism while peritoneal macrophages use a glycolytic pathway (156). The lysosomal

content of alveolar macrophages is higher than peritoneal macrophages but alveolar macrophages are considered less efficient in phagocytosis and lysis of bacteria (112,239). The Fc receptor avidity is lower in alveolar macrophages compared to peritoneal macrophages (172) and the former are less responsive to chemotactic agents (48,49). Another example of heterogeneity in macrophages obtained from different sites derives from work on Ia expression. The percentage of Ia positive macrophages within a body compartment is constant in normal animals although significant differences exist between compartments (15,36,51). Fifty percent of thymic (16), hepatic (174,176) and splenic macrophages (36) are Ia positive whereas 8-30% of peritoneal macrophages (192,246) and peripheral blood monocytes (6) possess this marker. The ability of these macrophages to present antigen also correlates with the percentage of Ia positive cells (51). Resident macrophages, which have low microbicidal activities, do not produce appreciable amounts of neutral proteinases and have negligible activity against tumor cells (2,55,211). Peritoneal cells elicited by intraperitoneal injection of thioglycolate (211), Corynebacterium parvum (143,158), phorbol myristate acetate (223), lymphokines (149), or phagocytosable particles (190) result in activated macrophages possessing high levels of antimicrobial and antitumor activity and high neutral proteinase levels.

The techniques previously described for isolating macrophages and monocytes have also been used to demonstrate heterogeneity among macrophage populations. Both density (173,194) and size (231) have been used to physically separate macrophage populations and demonstrate differences in functionality. Albumin, Ficoll and Percoll gradients have been used to fractionate macrophages and show a correlation between size and density with antigen-presenting capability and tumoricidal activity (160,173, 230,231,235). Counter-flow centrifugation elutriation has been used to demonstrate that large macrophages are more tumoricidal than smaller-sized phagocytes within the same macrophage population (140).

#### Monocyte Heterogeneity

The use of adherence for monocyte purification first indicated that monocytes were heterogeneous inasmuch as some, but not all, monocytes adhered to glass or plastic surfaces (89). The use of velocity sedimentation (8) and CCE (63,152,237,247) have demonstrated size and phenotypic differences in human monocytes. As first isolated by Norris et al., small human monocytes were reported to be Fc receptor negative and inactive in ADCC while the large monocytes were Fc receptor positive and active in ADCC (152). Subsequently, other investigators demonstrated differences between CCE isolated monocytes in lysosomal enzyme content (63), hexose monophosphate shunt activity

(195) and native tumoricidal activity (151). Of particular note, small monocytes possessed native tumoricidal activity while large monocytes did not and the former were more capable of being activated to higher tumoricidal levels in vitro than were large monocytes. Thus, heterogeneity appears to be present not only among the mature macrophages but also among the circulating monocyte precursors.

### Theories on Macrophage Heterogeneity

Accumulating data provide evidence for both intra-population and inter-population heterogeneity in the mononuclear phagocyte system. Current research has focused on the origin of this heterogeneity and its significance. At present two main concepts have been proposed to explain macrophage heterogeneity.

#### A. Vertical Heterogeneity: Maturation-Activation

The concept of vertical heterogeneity states that macrophage maturation with ensuing differentiation accounts for differences between and within macrophage populations. As the macrophage matures, different properties would be ascribed to different stages of differentiation. Under appropriate stimulation, mature macrophages would differentiate into the highly specialized activated macrophage capable of efficient microbicidal and tumoricidal functions. At a particular site, the macrophage pool would be expected to be in dynamic flux with its monocyte

precursors, thus providing a steady supply of unmaturation phagocytes.

Evidence to support this concept includes the following: First, monocytes derived from bone marrow continually renew macrophage populations at various body sites. Van Furth used tritiated thymidine labelled cells to demonstrate such monocyte traffic under steady state conditions (214). A different approach has been to use various marker enzymes. The enzyme, peroxidase, is present in monocytes and disappears when the cells mature (38). Peroxidase positive cells constitute a small but significant percentage of macrophages at various locations (23,221). Their number increases upon stimulation by various agents indicative of the increased influx of monocytes. The opposite situation pertains to the ectoenzyme 5'-nucleotidase (28). Second, monocytes are continuously differentiating into macrophages and a spectrum of functional characteristics can be expressed by any given macrophage (232).

Central to the argument for vertical heterogeneity is the requirement for only one type of committed macrophage stem cell in the bone marrow. That this may be so is supported by the bone marrow culture studies of Calamai et al. (29). They have shown that Ia positive and Ia negative macrophage colonies can be derived from a single bone marrow precursor. Depending upon the type of stimulation used, all colonies can be converted to the Ia

phenotype. This suggests that the committed macrophage stem cell is originally Ia negative and is induced to differentiate and express the Ia phenotype under appropriate immune stimulation.

B. Horizontal Heterogeneity: Population Diversity

The concept of horizontal heterogeneity states that different stem cells exist that give rise to monocytes with restricted functions and characteristics. Most studies in support of this concept have been carried out on macrophage colonies derived from bone marrow culture. In contrast to the data presented above supporting vertical heterogeneity, these studies have reported homogeneity within macrophages from discrete colonies but heterogeneity among the colonies themselves (71,126,134,182,234). The use of highly specific monoclonal antibodies has demonstrated major antigenic differences in macrophage colonies (182). The work of Metcalf also supports multiple stem cells in bone marrow as some CFU are capable of forming only macrophages while others can differentiate into either granulocytes or macrophages (134). The detection of a small but significant percentage of tissue macrophages not of monocyte origin with the capacity to replicate has been used as evidence for the existence of multiple macrophage stem cell precursors. This postulates the existence of at least 2 independent replicating progenitors as renewing the macrophage pool (114,116,117). In addition to this work

with macrophage colonies, the heterogeneous nature of circulating human monocytes has been used as evidence for macrophage stem cell heterogeneity. These proponents argue that macrophage precursors would use a common pathway, the circulation, to reach their destinations in the tissues and should be homogeneous. However, such homogeneity is not in fact observed, neither with respect to the properties already mentioned nor with monoclonal antibodies that have identified different populations of monocytes in human peripheral blood (88,169). Finally, it is apparent that the vast array of properties and functional diversity ascribed to the macrophage is unlikely to be possessed by every cell in the mononuclear phagocyte system. Indeed, the amount of cellular machinery necessary to produce such varied products in significant amounts argues strongly for functional stratification and therefore a heterogeneous macrophage population.

## SPECIFIC AIMS

At the present time, there is equal support for both the vertical and horizontal hypotheses of macrophage heterogeneity. Important to the interpretation of both hypotheses is an understanding of the characteristics and functions of the macrophage precursor cells: monoblasts, promonocytes and monocytes. Although culturing of monoblasts and promonocytes obtained from bone marrow has been successful in both human and mouse, it is well accepted that in vitro culture causes the maturation of these precursors into macrophages, thus distorting the differentiation-maturation steps which may occur. An alternative approach is to procure macrophage precursors in large numbers in an unaltered state from which characterization studies can be conducted. Human monocytes have been obtained in large numbers and high purity by the technique of counter-flow centrifugation elutriation. These monocytes displayed heterogeneity in physical characteristics and function. It is difficult to assess the significance of these findings in only one animal species. Furthermore, investigation of monocyte heterogeneity in human subjects is limited since most studies could be conducted only in vitro with little or no in vivo manipulation. The use of

an animal model would circumvent these problems and concurrently demonstrate monocyte heterogeneity in a species other than man.

The objectives of the experiments to be reported are to establish a method for isolating monocytes from a small laboratory animal, to demonstrate the functional integrity of the isolated monocytes and to investigate the possibility that monocytes of species other than man exist in different physical and functional states.

The following criteria should pertain to any method of isolating monocytes:

1. The starting monocyte preparation before separation should include all the monocytes found in the peripheral blood.
2. The separation method should yield cells in large enough quantities for characterization studies.
3. The separation method should be reproducible, showing consistency in the fractionation of the starting preparation.
4. The methods involved in separation should not cause alteration in the physical and functional properties of the monocytes.

The guinea pig was chosen for study because of the similarity of its hematopoietic system to that of man in both total and differential white blood cell counts (7,244). In addition, moderate quantities of guinea pig peripheral blood can be obtained without causing harm to

the animal or significant alteration in hemodynamics. Counter-flow centrifugation elutriation was chosen to separate guinea pig monocytes based upon the prior investigation of human monocyte heterogeneity using this technique.

## MATERIALS AND METHODS

### Mononuclear Cell Preparation

Seven male Hartley strain guinea pigs (750g) were anesthetized with a subcutaneous injection of 30 mg/kg Ketamine and 2 mg/kg Xylazine (78) after which a total of 150 ml of acid citrate dextrose (ACD) anticoagulated blood were obtained by cardiac puncture. The blood was diluted 1:1 with calcium and magnesium free Hank's balanced salt solution (HBSS) containing 100 mg EDTA/L. Twenty-one milliliters of diluted blood were placed in each of a series of 50 ml siliconized glass centrifuge tubes and 19 ml of Ficoll-Hypaque were introduced below the blood using an 18 gauge canula. After centrifugation at 400 x g for 40 minutes at 18°C (4), the interface layer was removed, diluted in HBSS, and the cells collected by centrifugation at 750 x g for 15 minutes at 4°C. The cells were washed twice and collected by centrifugation (150 x g for 10 minutes). Contaminating red blood cells were removed by exposure to buffered ammonium chloride (177) for 3 minutes after which the mononuclear cells were washed twice with the final washing being performed in elutriation medium consisting of calcium and magnesium free HBSS (pH 7.20) with 100 mg EDTA/L, 50 mg BSA/L and osmolality of 290 m

Osmos/L. Total cell counts were made by both a hemocytometer and a Coulter Counter Model ZF (Coulter Electronics, Hialeah, Fla.). Viability was determined by trypan blue dye exclusion.

#### Counter-Flow Centrifugation Elutriation (CCE)

Mononuclear cells ( $2 \times 10^8$ ) suspended in 5-10 ml of elutriation medium were injected into the inlet stream leading into the Beckman J-6B centrifuge equipped with a standard JE-6B elutriator rotor and chamber. A Masterflex peristaltic pump (Cole-Palmer Instruments, Chicago, IL) was equipped with a vernier potentiometer to provide precisely metered flow. The cells were loaded into the chamber at a flow rate of 10 ml/min, rotor speed of 3,000 RPM and a temperature of 4°C. Rotor speed was held constant and the cells eluted by changing the flow rate. In the first method, the flow rate was raised to where small cells began to exit the chamber and then sequential fractions of 100 ml each were collected at 1 ml/min increments in flow. In the second method, cells were eluted to exhaustion at each of 2 predetermined flow rates. In both techniques, cells remaining in the elutriator chamber were collected by continuing medium flow after stopping the rotor. Each cell fraction was collected by centrifugation, washed twice in RPMI 1640 and counted, after which viability was determined. Slides were prepared using a cytocentrifuge (Shandon Southern Instruments, Sewickley, Pa.) at  $5 \times 10^4$

cells per slide in 50% fetal calf serum. Cell differentials were done on 500 cells stained with Wright-Giemsa (Camco Quick Stain, Am. Scientific Products, Ocala, FL). A Leitz Dialux 20 microscope with orthomat camera was used to photograph the stained slides.

### Cell Volume Analysis

Cell volume was determined with a Particle Data System 80 XY Electrozone Celloscope integrated with the REX 604 software program (Particle Data Systems, Chicago, IL). This system provides a computer smoothed volume plot based upon total cells in the peak channel.

### Histochemical Stains

The non-specific esterase stain was modified from that described by Yam et al. (245). Formalin-acetone fixed cytocentrifuge slides were reacted for 50 minutes at 37°C with alpha naphthyl acetate, then washed in tap water, and counter-stained for 3 minutes in 1% methyl green. Guinea pig monocytes unlike human monocytes stained weakly at room temperature but reacted well at 37°C to yield a reddish-brown reaction product. Acid phosphatase staining was done using the fast garnett GBC method with Napthol AS-BI phosphoric acid salt (Sigma Chemical Co., St. Louis, MO) as substrate and scored for the intensity of orange-red reaction product found within the cytoplasm (115). Peroxidase staining was performed either as described by

Kaplow (95,96) at pH 6.0 with maximal staining after 1 minute incubation or as modified by Meltzer (130) using a 2 minute reaction at pH 7.0. The slides were counter-stained with Giemsa and the reaction product scored. Guinea pig peripheral blood smears were always run as controls for the above histochemical stains. Staining intensity was graded on a 0-4+ scale of intensity and monocytes having less than a 1+ reaction were considered negative.

#### Adherence

Cells suspended in alpha-MEM containing 10% heat inactivated guinea pig serum were tested for adherence using four methods. First, cells were allowed to adhere to untreated 75 cm<sup>2</sup> plastic tissue culture flasks (Costar, Cambridge, MA.) or second, to flasks coated with a micro-exudate from a previous culture of a BHK-21 cell line as described by Ackermann and Douglas (1). In both methods, non-adherent cells were removed by repetitive washing as judged by inverted phase microscopy after which the adherent cells were recovered by vigorous agitation following a 15 minute exposure at 37°C to 10 ml of medium containing 5mM/L EDTA. Third, 2 ml of 1% type I bovine gelatin (Sigma Chemical Co., St. Louis, MO) were added to 35 X 10 mm tissue culture plates (Falcon Plastics, Oxnard, CA) and refrigerated overnight. After removing excess gelatin, the plates were overlaid with 2 ml of fresh heparinized guinea pig plasma or purified human fibronectin

(Sigma Chemical Co., St. Louis, MO) at 37°C for 30 minutes (67). After washing three times, the various cell fractions were added for 1, 4, or 8 hours. Then non-adherent cells were washed away and adherent cells detached by overnight incubation and mild agitation. Fourth, up to  $1 \times 10^8$  cells were passed over nylon wool columns prepared as described by Weinblatt et al. (236) using 3 ml columns containing 0.2 grams of sterile, acid-washed nylon wool. After incubation, non-adherent cells were eluted with 100 ml of medium and adherent cells recovered by exposing the column for 15 minutes to medium containing 5mM/L EDTA and mechanically expressing the cells from the column. All adherence procedures were performed at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. In some experiments, lipopolysaccharide derived from Salmonella typhimurium (Difco Laboratories, Detroit, MI) was used to activate the cells prior to adherence. The percentage of adherence was calculated from the ratio of the number of monocytes recovered from the adherent fraction or the number depleted from the non-adherent fraction to the total number of applied monocytes. The reported percentage of monocyte adherence is the higher of these two calculations although in most instances the two calculations were identical.

#### Percoll Gradients

Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) was adjusted to a specific gravity of 1.070, pH of 7.20 and

12 ml used to generate a continuous gradient in a 13 x 100 mm polycarbonate tube by centrifuging at 30,000 x g in a fixed angle rotor for 20 minutes at 18°C in a Sorval RC-2B centrifuge. Cells from the 24A ( $50 \times 10^6$ ), 24B ( $6 \times 10^6$ ) and 28 ml/min ( $10 \times 10^6$ ) were mixed with 1 ml of Percoll removed from the bottom of the gradient and then underlaid in the same gradient tube. These gradients along with a control gradient containing density marker beads (Pharmacia Fine Chemicals, Piscataway, NJ) were centrifuged at 400 x g for 20 minutes at 18°C. Mean cell densities were determined by comparing the distance migrated by cells to that of the marker beads. The various cell fractions were removed and washed twice before analysis for cell number, viability and size.

### Phagocytosis

Non-immune phagocytosis was performed using carbon particles and albumin-coated polystyrene latex spheres. Carbon particles (suspension lot C-11-1431a, from Gunther-Wagner, Hanover, West Germany) were diluted to 25 mg/100 ml in saline and 15  $\mu$ l added to  $2 \times 10^6$  mononuclear cells in a total volume of 1.0 ml of RPMI 1640 with 10% fetal bovine serum. Fluorescent labeled latex particles of 0.7 micron diameter (Covaspheres FX Particles) were obtained from Covalent Technology Corp., Ann Arbor, MI. Covalent bonding of protein to the activated bead surface was performed by adding 50  $\mu$ l of Covaspheres to 0.5 ml of HBSS containing

0.1 mg BSA/ml. Bonding was nearly instantaneous and the albumin coated beads were collected by centrifugation, washed twice in HBSS and resuspended in 7 ml of HBSS containing 10% fetal bovine serum. The phagocytosis assay was performed in 12 x 75 mm polypropylene tubes at 37°C by exposing  $2 \times 10^6$  mononuclear cells in 0.5 ml to an equal volume of bead suspension. In both assays, non-ingested particles were removed by gently centrifuging the cells (100 x g, 8 minutes) through 1 ml of fetal bovine serum. Cytocentrifuge slides were prepared from the cell pellet and stained with Wright-Giemsa. The intensity of carbon phagocytosis per 100 phagocytes was scored on a 0 to 4+ scale using the oil immersion objective of a light microscope. The fluorescent labeled beads were visualized using a Zeiss epifluorescent microscope and the number of beads counted in 100 phagocytes. In both assays, cell suspensions also were placed directly on glass slides and viewed using a combination of tungsten and ultraviolet light with a Zeiss epifluorescent microscope. This method helped distinguish those cells which had beads bound to their surfaces (rosettes) as opposed to those which had engulfed them.

The ability of monocytes to participate in antibody-dependent phagocytosis was assessed with antibody coated sheep erythrocytes. Sheep erythrocytes sensitized with rabbit anti-sheep red blood cell immunoglobulin (anti-SRBC IgG from Cordis Laboratories, Miami, FL) were added to

guinea pig mononuclear cells suspended in RPMI 1640 with 10% fetal bovine serum at a concentration of 100 erythrocytes per monocyte. Following light centrifugation (150 x g for 3 minutes) and incubation at 37°C in a 5% CO<sub>2</sub> incubator, the uningested erythrocytes were lysed by exposure to ice cold buffered ammonium chloride for 5 minutes. The remaining mononuclear cells were washed once in RPMI 1640 and cytocentrifuge slides prepared after resuspension in RPMI 1640 containing 50% fetal bovine serum. The percentage of monocytes phagocytizing erythrocytes as well as the number of RBC's phagocytized per 100 monocytes was scored by the use of oil immersion light microscopy on Wright-Giemsa stained preparations.

Candida albicans (human blood isolate) was cultured overnight in Saboraud's broth on a 180 RPM shaker at 37°C. For opsonization,  $5 \times 10^7$  organisms in 1 ml of RPMI 1640 were incubated on ice with 1 ml of fresh guinea pig serum for 15 minutes. Opsonized and non-opsonized Candida albicans were added to the various mononuclear fractions at a 10:1 ratio of organisms to phagocytes for various time periods at 37°C. Phagocytosis was evaluated using cytocentrifuge preparations stained with Wright-Giemsa and reported as the number of Candida ingested per 100 phagocytes.

For some experiments, the C3 component of guinea pig sera was neutralized by incubating rabbit anti-guinea pig C3 (Cappell Laboratories, Cochranville, PA) with fresh

guinea pig sera for 30 minutes at 37°C. Immune precipitate was removed by ultracentrifugation (Beckman airfuge, Beckman Instruments, Palo Alto, CA). The C3 component was no longer demonstrable when this sera was reacted against anti-C3 by Oucaterlony analysis.

#### Guinea Pig Anti-Sheep Erythrocyte Antibody

Autologous IgG against sheep red blood cells (SRBC) was prepared in 3 guinea pigs by injecting intramuscularly and subcutaneously 0.1 ml of 20% SRBC's in complete Freund's adjuvant (Difco Laboratories, Detroit, MI). An additional 0.1 ml of cell suspension was administered subcutaneously 14 days later. After 5 weeks, blood was drawn by cardiac puncture from which serum was obtained and heat-inactivated for 30 minutes at 56°C. Guinea pig IgG was purified using ammonium sulfate fractionation. Then, serial dilutions of this antibody were incubated for 30 minutes at 37°C in a 1:1 volume ratio with a 5% suspension of washed SRBC's in 0.01 M EDTA gelatin-veronal buffer (EDTA-GVB from Cordis Laboratories, Miami, FL). After washing three times, the pellets were resuspended and evaluated for agglutination under an inverted phase microscope (Swift Instruments, Inc., San Jose, CA) using the 40 x phase objective. The smallest dilution of antibody which did not produce detectable clumping was 1:100.

### Fc Receptor Assay

Fresh sheep red blood cells (SRBC) were collected in Alsever's solution and washed three times in cold phosphate buffered saline. Rabbit anti-sheep red blood cell immunoglobulin (anti-SRBC IgG from Cordis Laboratories, Miami, FL) was determined to have a subagglutinating titer of 1/2000. To 1 ml of 5% SRBC in EDTA-GVB was added an equal volume of a subagglutinating dilution of anti-SRBC IgG and the mixture incubated at 37°C for 30 minutes. Fresh ox (bovine), guinea pig and human red blood cells were sensitized with anti-erythrocyte immunoglobulin in a similar manner. Rabbit anti-ox (bovine) erythrocyte immunoglobulin (subagglutinating titer 1:500) and rabbit anti-guinea pig erythrocyte immunoglobulin (subagglutinating titer 1:5000) were obtained from Cappel Laboratories, Cochranville, PA. Human anti-Rh<sub>0</sub>D immunoglobulin (Dade Diagnostics, Miami, FL) reacted optimally at a titer of 1:50 against O positive human red cells. After reacting with antibody, the sensitized erythrocytes were washed three times in EDTA-GVB buffer, resuspended in HBSS containing 2% BSA, counted and adjusted to  $4 \times 10^7$  erythrocytes/ml.

The assay for Fc receptors was based upon the capacity of the monocytes to form rosettes with antibody sensitized erythrocytes of guinea pig, ox and human. In 12 x 75 mm polypropylene tubes, 100  $\mu$ l of sensitized erythrocytes were added to 100  $\mu$ l of mononuclear cells ( $4 \times 10^6$  cells/ml)

to yield a 10:1 ratio of erythrocytes to monocytes. The suspension was vortexed lightly, centrifuged at 100 x g for 3 minutes and incubated for 30 minutes at 37°C. Then the pellet was gently resuspended and 15  $\mu$ l placed on a glass slide under a coverslip. The percentage of monocytes binding 0-2, 3-4 or 5 or more erythrocytes was determined in a 200 monocyte count using oil immersion phase contrast with greater than 2 erythrocytes bound per monocyte considered an Fc receptor positive cell. Phase contrast highlighted the granulated cytoplasm found in monocytes and allowed positive identification of these cells. In addition, cytocentrifuge slides were made, stained with Wright-Giemsa and the percentage of rosette-positive monocytes determined. In all assays, a positive control was run concurrently consisting of proteose-peptone elicited peritoneal guinea pig macrophages which were routinely greater than 90% Fc receptor positive.

Previously, Fc receptors on guinea pig monocytes were determined on cells after 48 hours of culture (23). Accordingly, mononuclear cell preparations in some experiments were incubated at 37°C and 5% CO<sub>2</sub> for various time periods before determination of Fc receptors. For culture, mononuclear cells were diluted to a concentration of  $1 \times 10^6$  cells/ml with RPMI containing 10% guinea pig serum, 10% horse serum, 10 mM HEPES buffer, 50  $\mu$ g/ml gentamycin, 10,000 U penicillin, and 5,000 U streptomycin. Ten milliliters of cell suspension were cultured in 30 ml

capacity Teflon FEP culture bottles (Nalge Corp., Rochester, NY) and Fc receptor analysis performed as described.

#### Antibody-Dependent Cellular Cytotoxicity (ADCC)

Fresh sheep erythrocytes were sensitized with a subagglutinating titer of rabbit anti-SRBC immunoglobulin as described for the determination of Fc receptors. When used as target cells in ADCC, they were labeled with radioactive chromium by exposing  $4 \times 10^7$  cells in the bottom of a centrifuge tube to 200  $\mu$ Ci of radioactive chromium (250-500 mCi/mg chromium, Amersham, Arlington Heights, IL) for 1 hour at 37°C. After washing twice in HBSS and once in RPMI, the labeled cells were resuspended in ADCC medium (RPMI 1640 with 10% guinea pig serum, 10 mM HEPES buffer, 50  $\mu$ g/ml gentamycin, 10,000 U penicillin and 5,000 U streptomycin). Labeled cells averaged 10,000 CPM/ $10^6$  SRBC using a Beckman 300 Gamma Counter (Beckman Instruments, Fullerton, CA). Effector cells in 1 ml of ADCC media were dispensed into 12 x 75 mm polypropylene tubes and 100  $\mu$ l of target cells added ( $2 \times 10^5$  SRBC). Non-specific isotope release was determined by adding 100  $\mu$ l of target cell suspension to 1 ml of assay medium. All tests were done in triplicate. The assay was performed by gently centrifuging the cell suspension (100 x g for 3 minutes) and incubating the pellets at 37°C in a 5% CO<sub>2</sub> incubator. At the conclusion of the assay (8 or 16 h), the

tubes were gently agitated, the cells centrifuged at 400 x g for 5 minutes and 550  $\mu$ l of the supernatant removed and counted in a Beckman 300 Gamma Counter. The percent  $^{51}\text{Cr}$  release was determined using the formula:

$$\% \text{ } ^{51}\text{Cr} \text{ release} = \frac{\text{Experimental CPM} - \text{Control CPM}}{\text{Total CPM} - \text{Control CPM}} \times 100$$

### Tumoricidal Assay

The P-815 mastocytoma cells were maintained in ascites form by serial IP transplantation of  $2 \times 10^6$  cells in syngeneic DBA/2 mice. Tumor cells were harvested 3 to 5 days after transplantation and cultured overnight at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ . Culture medium consisted of RPMI 1640 with 10% guinea pig serum, 10mM HEPES buffer, 50  $\mu\text{g}/\text{ml}$  gentamycin, 10,000 U penicillin and 5,000 U streptomycin. Tumor cells were labeled for 4 hours with 0.5  $\mu\text{Ci}/\text{ml}$   $^{125}\text{-iododeoxyuridine}$  ( $^{125}\text{Iudr}$  from Amersham Corp., Arlington Hts, IL), washed three times in culture medium and counted. Viability was determined by trypan-blue dye exclusion. Labeled target cells averaged 4,000 CPM/ $10^4$  cells with greater than 95% viability by trypan blue dye exclusion. Effector cells were washed twice in RPMI 1640, resuspended in culture medium and counted. Differential cell counts were made on cytocentrifuge slides stained with Wright-Giemsa. A constant number of tumor cells ( $1 \times 10^4$  in 100  $\mu\text{l}$ ) was added to 1 ml of effector cells in 12 x 75 mm polypropylene tubes at varying effector:target (E:T) ratios

based on monocyte number. Controls consisted of radio-labeled tumor cells added to 1 ml of culture medium. All tests were done in triplicate. At the conclusion of the assay, the tubes were gently mixed, centrifuged at 400 x g for 5 minutes and 550 $\mu$ l of the supernatant removed.

Percent isotope release was calculated using the formula:

$$\% \text{ } ^{125}\text{I} \text{ release} = \frac{\text{Experimental CPM} - \text{Control CPM}}{\text{Total CPM} - \text{Control CPM}} \times 100$$

## RESULTS

### Isolation of Mononuclear Cells

Ficoll-Hypaque of specific gravity 1.079 is used routinely to isolate human monocytes in high yield but the same preparation collected less than 25% of guinea pig monocytes at the interface layer. Table 1 presents data on the recovery of guinea pig monocytes using Ficoll-Hypaque of increasing specific gravity. From this analysis, we concluded that a high specific gravity of 1.101 was required to routinely recover greater than 95% of the monocytes applied to the gradient. Although red cell contamination increased with the higher density Ficoll-Hypaque, these cells were easily removed by ammonium chloride lysis. In addition, granulocytes contaminated the mononuclear cell fraction when high density Ficoll-Hypaque gradients were prepared in polypropylene tubes. This problem was corrected by using siliconized glass tubes in place of polypropylene tubes. As shown in Table 2, the absolute number of granulocytes was reduced by using siliconized tubes whereas mononuclear cell numbers remained unchanged.

TABLE 1. INCREASED RECOVERY OF GUINEA PIG MONOCYTES WITH INCREASED SPECIFIC GRAVITY OF FICOLL-HYPAQUE

Ficoll-Hypaque Specific Gravity	Monocyte Recovery	Monocyte Concentration
1.078	25 ± 6	2.5 ± 1.0
1.085	57 ± 3	6.0 ± 2.0
1.095	76 ± 4	8.0 ± 1.0
1.101	95 ± 2	12.0 ± 2.0

Results are the mean ± S.E. of 3-6 experiments.

TABLE 2. COMPARISON BETWEEN POLYPROPYLENE AND SILICONIZED GLASS TUBES  
WITH RESPECT TO GRANULOCYTE CONTAMINATION OF FICOLL-HYPAQUE  
PREPARATIONS

Determination	Recovered Cells x 10 <sup>5</sup>	
	Polypropylene Tubes	Siliconized Glass Tubes
Total Cells	280	220
Granulocytes	52 (18.3%)	3.5 (1.6%)
Monocytes	22	19
Lymphocytes	200	200

Mean data from three experiments using post Ficoll-Hypaque separated cells at a specific gravity of 1.101.

### Counter-Flow Centrifugation Elutriation (CCE)

At a loading flow rate of 10 ml/minute and rotor speed of 3,000 RPM, the mononuclear cells remained suspended in the chamber while platelets and residual red cells were purged. No mononuclear cells were collected until the flow rate reached 24 ml/minute. Table 3 presents the profile of cells eluted at sequential 1 ml/minute increases in flow above 24 ml/minute showing a progressive decrease in lymphocytes and an increase in monocytes. The 28 ml/minute fraction had the highest monocyte purity (70%) and yield (54%). The residual cells purged from the chamber after stopping centrifugation were designated rotor-off (R/O) cells. Monocytes accounted for 46% of the R/O cells along with large lymphocytes, Kurloff cells and some granulocytes.

Volume determinations showed that the 24 ml/minute fraction resembled the major post-Ficoll-Hypaque peak having an identical modal volume of  $153\mu^3$ . Analysis of the fractions collected at each flow increment demonstrated a gradual decrease in the  $153\mu^3$  peak and the appearance of a  $317\mu^3$  peak starting at 25 ml/minute. This latter peak corresponded to the shoulder of the original post-Ficoll-Hypaque preparation. The 28 ml/minute fraction, which had the highest monocyte percentage, showed the lowest percentage of cells in the  $153\mu^3$  range and the highest percentage in the  $317\mu^3$  range. The R/O fraction had a modal volume slightly greater than the 28 ml/minute

fraction ( $354\mu^3$ ). We concluded that the size profile of the cells was a useful indicator of the differential cell composition with a peak at  $153\mu^3$  being characteristic of lymphocytes and  $317\mu^3$  of monocytes.

In the next series of experiments, separation was achieved using only two flow rates (Table 4). As in the previous method, the first collection was made at 24 ml/minute but this flow rate was now held constant until 400 ml had been collected. For purposes of analysis, this collection was divided into two fractions of 200 ml each. The first 200 ml (24A fraction) contained 92% lymphocytes, 5% monocytes and 3% Kurloff cells. It displayed a volume peak at  $153\mu^3$  and had a low shoulder representing cells with volumes greater than  $300\mu^3$  (Figure 1). After 200 ml had been collected, very few cells exited the chamber so that the second 200 ml fraction contained less than  $1 \times 10^7$  total cells. This fraction, designated 24B, contained cells which displayed a bimodal size profile consistent with the numbers of lymphocytes (28%) and monocytes (64%) found by differential cell counting (Figure 1). A second collection was made at a flow rate of 28 ml/minute and all cells capable of eluting were collected in a total volume of 200 ml. This fraction had an average monocyte purity of 31% (Table 4). Modal volume analysis of this fraction displayed a single narrow peak at  $317\mu^3$  and failed to detect any cells with a peak volume of  $153\mu^3$  (Figure 1). The R/O cells were similar in differential

composition and volume analysis to those obtained when elutriation was carried out at 1 ml/minute increases in flow rate.

The morphology of the monocytes obtained by the two techniques of elutriation was identical (Figures 2-8). Monocytes collected at 24 and 28 ml/minute possessed an eccentric, reniform nucleus, a glassy sky-blue cytoplasm when stained with Wright-Giemsa and a slight cytoplasmic vacuolization. The R/O monocytes were larger, possessed a centrally located large nucleus and had a pyroninophilic cytoplasm with vacuolization. To further delineate the significance of the cells contained in the 24B fraction, 24A and 28 ml/min cells were reconstituted and re-eluted using the two flow rate technique (Table 5). A 24B fraction of identical total cell number was again recovered. This fraction had a higher percentage of monocytes than the original 24B fraction with a modal volume identical to that obtained with the 28 ml/min ( $317 \mu^3$ ).

In comparison to incremental flow rates, the two flow rate procedure yielded greater purity of the intermediate sized monocytes and was easier to perform and control. For these reasons, the two flow rate procedure was utilized in subsequent studies.

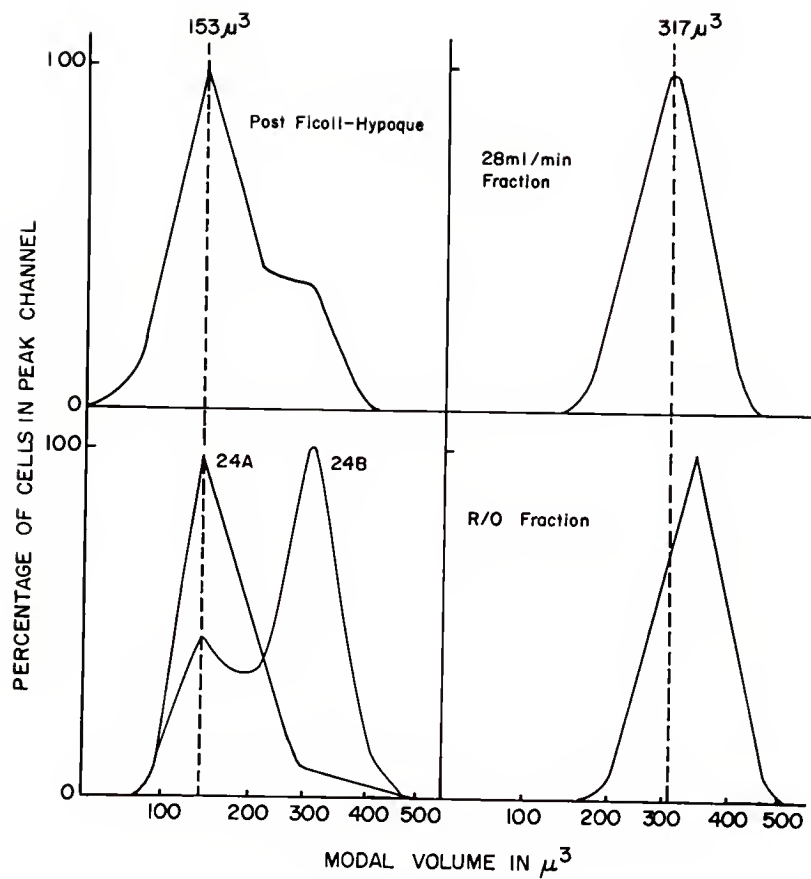


FIGURE 1.

Volume analysis of guinea pig mononuclear cells separated by counter-flow centrifugation elutriation. Drawing reproduced from an actual computer smoothed volume plot obtained from a Particle Data System pulse height analyzer.

TABLE 3. COUNTERFLOW CENTRIFUGATION ELUTRIATION OF GUINEA PIG MONONUCLEAR CELLS USING 1 ML./MINUTE INCREMENTS IN FLOW RATE

Fraction	Relative Monocyte Distribution	Modal Volume ( $\mu^3$ )	Total Cells ( $\times 10^6$ )	Leukocyte Differential %	
				Lymphocytes	Kurloff Cells Monocytes
Peripheral blood				$45 \pm 3$	$<0.5$ $3.2 \pm 1.6$
Pre-elutriation	100%	153,317	200	$85 \pm 6$	$3.2 \pm 0.5$ $12 \pm 2.1$
Post-elutriation <sup>a</sup>					
24 ml/minute	15%	153	$127 \pm 2$	$96 \pm 2$	$2.4 \pm 1.9$ $2.0 \pm 0.6$
25 ml/minute	10%	153,300	$22 \pm 2$	$77 \pm 6$	$3.8 \pm 1.1$ $19 \pm 7$
26-27 ml/minute	10%	317	$16 \pm 3$	$30 \pm 2$	$10.1 \pm 1.5$ $60 \pm 6$
28 ml/minute	54%	317	$4 \pm 1$	$25 \pm 4$	$4.8 \pm 2.8$ $70 \pm 7$
Rotor-off (R/O)	11%	354	$5 \pm 1$	$33 \pm 7$	$9.9 \pm 4.2$ $46 \pm 9$

Data are reported as mean  $\pm$  standard error on 10 determinations. The peripheral blood white cell count was  $7.1 \pm 0.6$  million/ml. The pre-elutriation fraction was prepared by Ficoll-Hypaque separation of blood.

a. Each elutriation fraction was collected in a total volume of 100 ml. Rotor speed was constant throughout at 3,000 RPM.

TABLE 4. COUNTERFLOW CENTRIFUGATION ELUTRIATION OF GUINEA PIG MONONUCLEAR CELLS USING A TWO FLOW RATE METHOD OF FRACTIONATION.

Fraction	Relative Monocyte Distribution	Modal Volume $\mu^3$	Total Cells ( $\times 10^6$ )	Leukocyte Differential %		
				Lymphocytes	Kurloff Cells	Monocytes
Pre-elutriation	100%	153	200.0	$85 \pm 6$	$3.2 \pm 0.5$	$12 \pm 2.1$
Post-elutriation <sup>a</sup>						
24 ml/minute						
0-200 ml	33%	153	$155 \pm 4$	$92 \pm 1$	$3.1 \pm 0.5$	$5 \pm 0.7$
200-400 ml	19%	153,300	$6.4 \pm 0.6$	$28 \pm 6$	$7.5 \pm 1.6$	$64 \pm 5.9$
28 ml/minute						
0-200 ml	42%	317	$11.0 \pm 1.5$	$8 \pm 1$	$7.4 \pm 0.9$	$81 \pm 2.3$
Rotor-off (R/O)	7%	354	$3.7 \pm 0.5$	$17 \pm 3$	$15.7 \pm 1.4$	$43 \pm 4.9$

Data are reported as mean  $\pm$  standard error on 10 determinations. The pre-elutriation fraction was prepared by Ficoll-Hypaque separation of whole blood and elutriation was carried out using  $2 \times 10^8$  cells.

a. Rotor speed was constant throughout at 3,000 RPM.

TABLE 5. RE-ELUTRIATION OF 24A AND 28 ML/MIN OCE FRACTIONS

Fraction	First Elutriation <sup>a</sup>			Re-elutriation <sup>b</sup>		
	Cell Number	Monocyte Percentage	Monocyte Number (10 <sup>6</sup> )	Cell Number (10 <sup>6</sup> )	Monocyte Percentage	Monocyte Number (10 <sup>6</sup> )
Loaded cells						
(unfractionated)	136	17	23	100	19	19
24A	100	6	6	71	8	6
24B	5	64	3	4	73	3
28	15	91	13	12	81	10

<sup>a</sup>The first elutriation was performed using a 2 flow rate method of elutriation. The loaded cells consisted of  $136 \times 10^6$  post Ficoll-Hypaque mononuclear cells.

<sup>b</sup>The first 200 ml collection fraction at 24 ml/min (24A) and the 28 ml/min fraction (200 ml) were centrifuged, recombined and re-eluted under the same conditions as the original elutriation. Results represent the mean of 2 experiments.

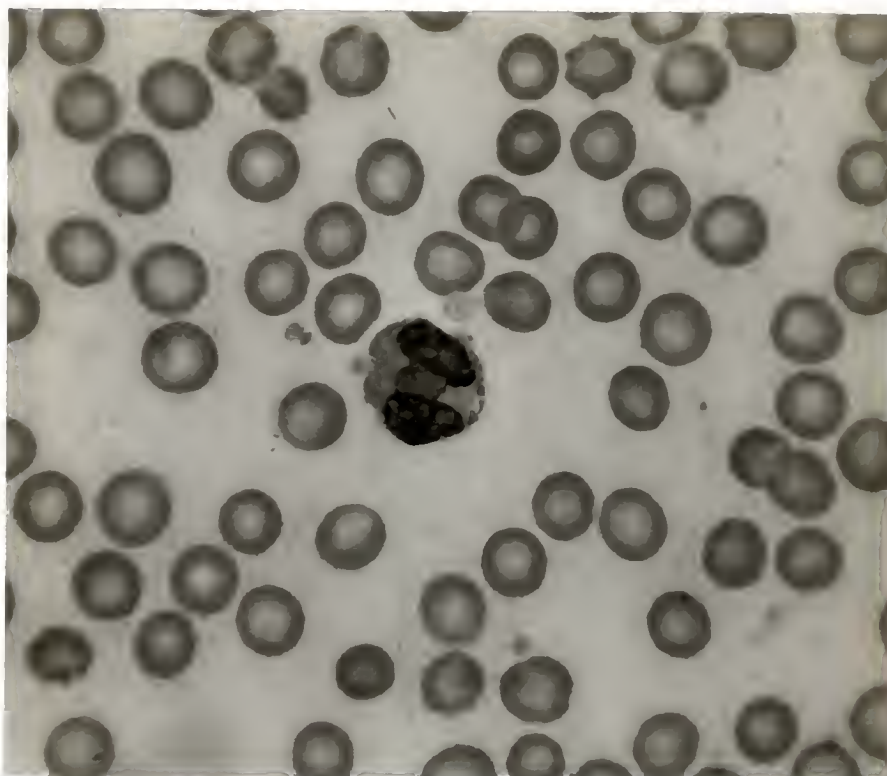


FIGURE 2.

Morphology of guinea pig monocytes (peripheral blood smear x 2500). The monocyte in this peripheral blood smear is indicative of the morphology of the majority of guinea pig monocytes. The nucleus is convoluted and classically reniform and the cytoplasm is finely granulated with well-defined cell borders.

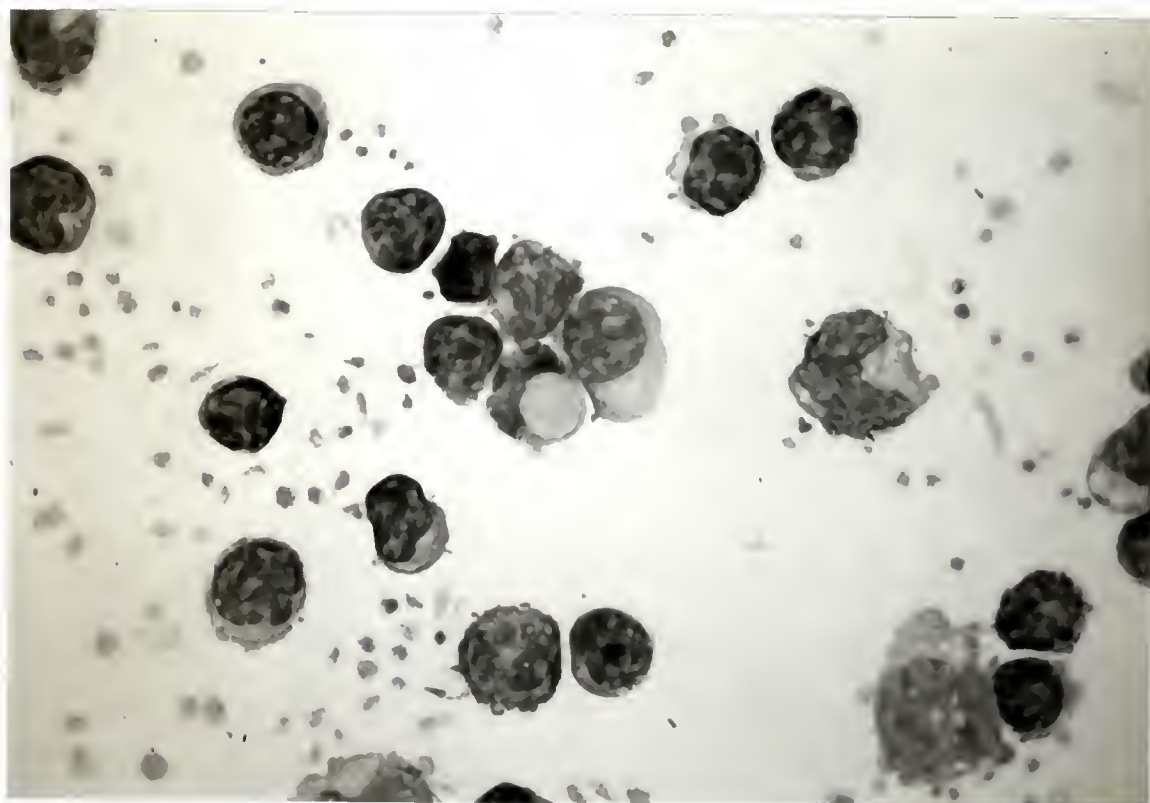


FIGURE 3.

Morphology of guinea pig mononuclear cells separated by counter-flow centrifugation elutriation (x 2500).

Post Ficoll-Hypaque mononuclear cell preparation before elutriation. The majority of cells are lymphocytes but monocytes and Kurloff cells are evident. Large numbers of platelets contaminate the preparation before elutriation.

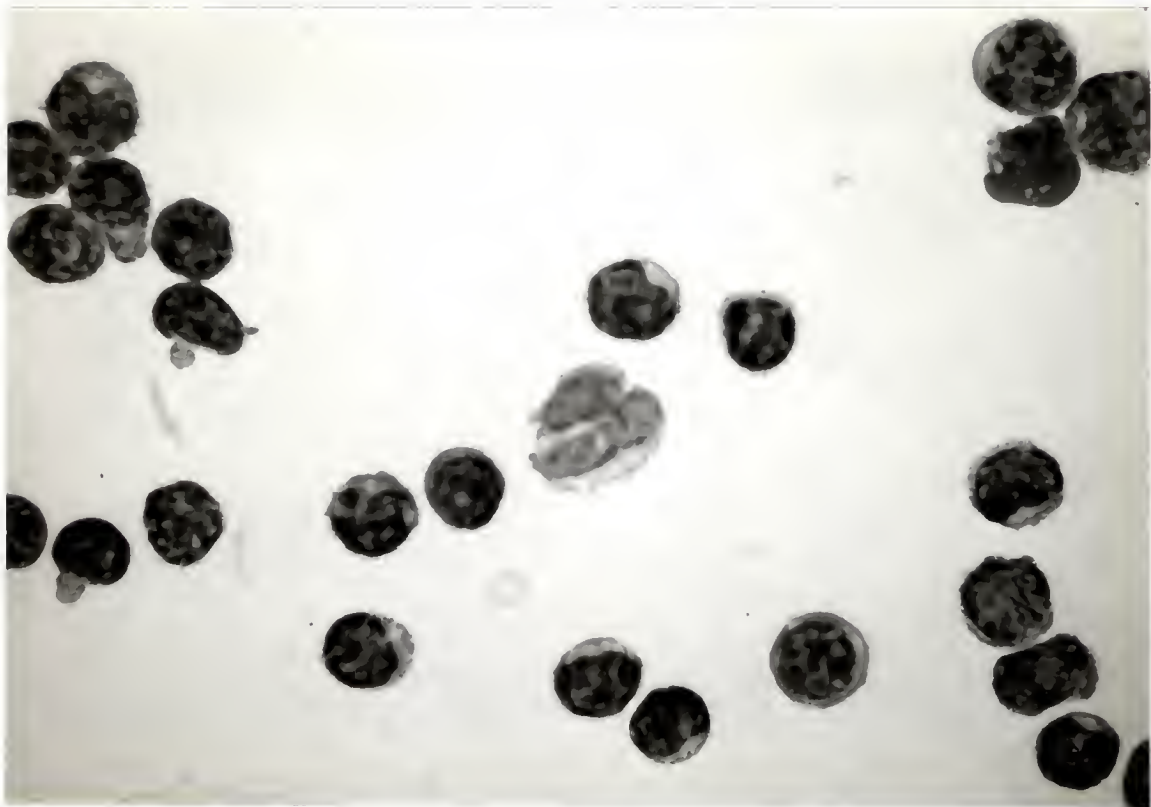


FIGURE 4.

Morphology of guinea pig mononuclear cells separated by counter-flow centrifugation elutriation (x 2500). The 24A fraction (24 ml/minute, 0-200 ml collection). This fraction is composed largely of lymphocytes. In the center is a small monocyte with its typical reniform nucleus and scant cytoplasm.

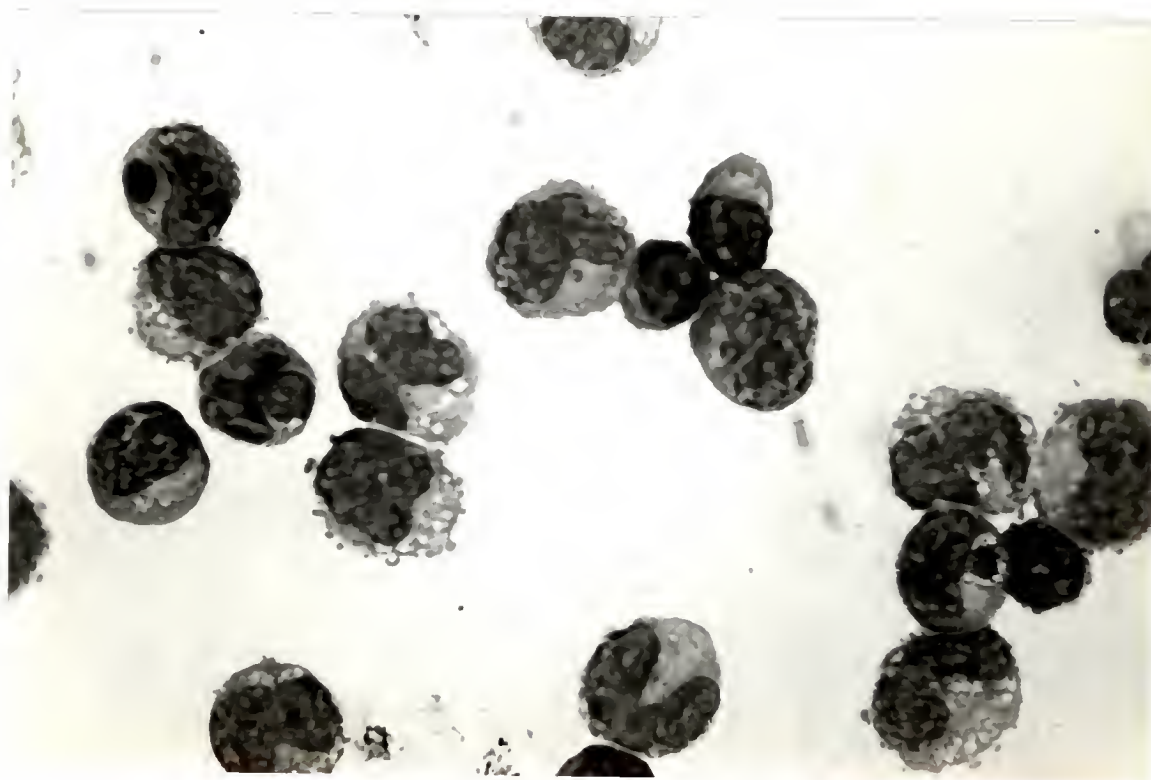


FIGURE 5. Morphology of guinea pig mononuclear cells separated by counter-flow centrifugation elutriation (x 2500). The 24B fraction (24 ml/minute, 200-400 ml collection). This fraction has predominantly monocytes with lesser numbers of lymphocytes and Kurloff cells.



FIGURE 6. Morphology of guinea pig mononuclear cells separated by counter-flow centrifugation elutriation (x 2500). The 28 ml/minute fraction. This fraction contains intermediate-sized monocytes characterized by a reniform nucleus and slight vacuolization.

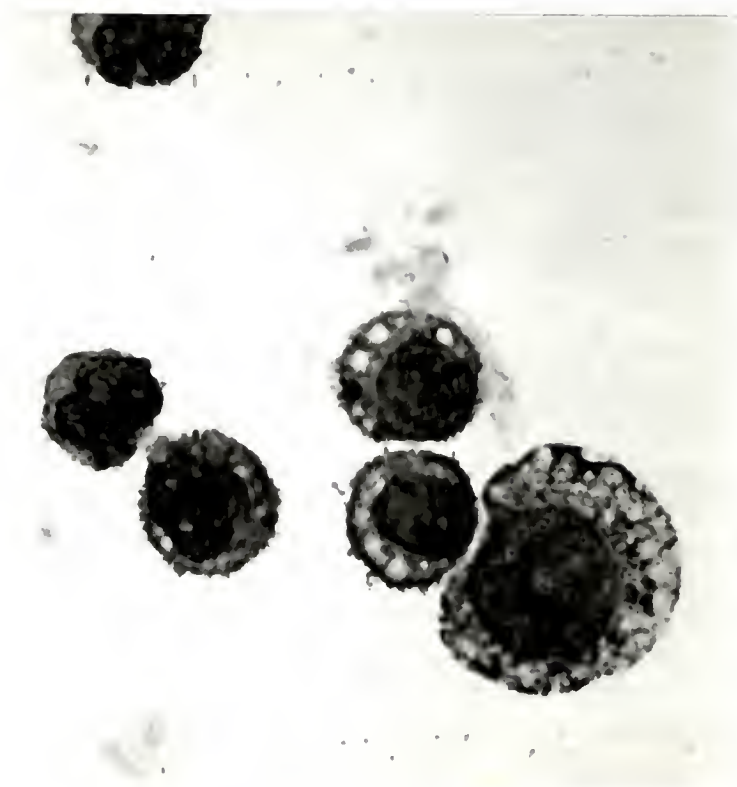


FIGURE 7.

Morphology of guinea pig mononuclear cells separated by counter-flow centrifugation elutriation (x 2500).

The rotor-off (R/O) fraction. This fraction contains large monocytes which have a spherical nucleus, abundant cytoplasm and heavy vacuolization. Although not shown, an occasional monocyte is binucleated.

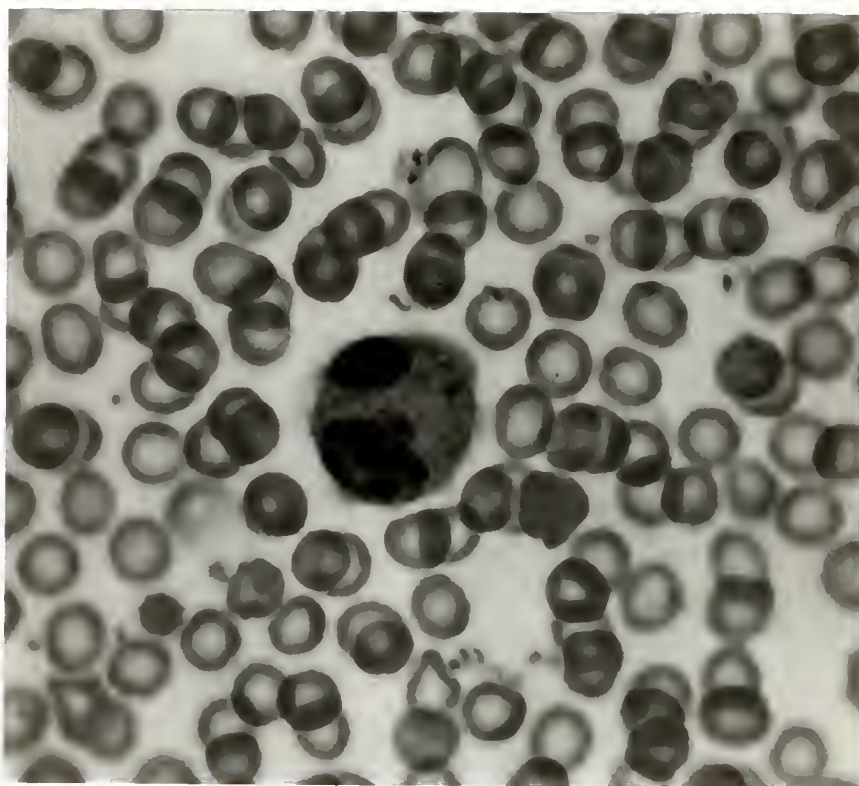


FIGURE 8.

Morphology of rotor-off (R/O) monocytes of guinea pig peripheral blood (x 2500). R/O monocytes constitute an extremely small percentage of the total blood leukocytes in the guinea pig (0.14%). As depicted, this cell may be binucleated with the nucleus more spherical than those of the majority of guinea pig monocytes. The cytoplasm is also more abundant and more heavily granulated with irregular cell borders.

### Histochemical Staining

Post-elutriation monocytes were analyzed for non-specific esterase, acid phosphatase, and peroxidase. All guinea pig monocytes were non-specific esterase positive with monocytes of the 24A fraction having a 1+ localized reaction, the 24B fraction a 1+ to 2+ localized reaction, the 28 ml/minute fraction a 2+ diffuse reaction and R/O monocytes a 4+ mixed reaction. Acid phosphatase was graded 2+ in R/O monocytes and in monocytes of the 24B and 28 ml/minute fractions but zero in monocytes of the 24A fraction. Peroxidase was negative in monocytes from all fractions using the standard Kaplow staining technique (95,96), although a few cells of the 28 ml/minute and R/O fractions showed a  $\pm$  perinuclear reaction. However, 40% of post-Ficoll Hypaque guinea pig monocytes were positive (1+ to 2+) for peroxidase using Meltzer's modified peroxidase method (130). With this technique, 41% of the 24A, 45% of the 24B and 33% of the 28 ml/minute monocytes were positive displaying a 1+ to 2+ reaction. Only 4% of the R/O monocytes were positive.

### Adherence Characteristics of Guinea Pig Monocytes

Adherence of human and murine monocytes to plastic, nylon wool, microexudate or fibronectin (plasma)-coated plates has been used either to purify monocytes (1,67,110, 236) or to deplete them from preparations of lymphocytes (93,145) as well as natural killer (NK) cells (81).

Accordingly, the capacity of guinea pig monocytes to adhere to similar substrates was investigated, searching not only for a method to increase monocyte purity, but also for differences between the monocyte fractions.

Post-Ficoll-Hypaque guinea pig monocytes or monocytes contained within the 24A, 24B and 28 ml/minute collections did not adhere to plastic tissue culture flasks or to BHK-21 microexudate coated plates (Table 6). Addition of gram negative bacteria (Pseudomonas fluorescens) or 5  $\mu$ g/ml lipopolysaccharide caused monocyte adherence within two hours demonstrating that adherence in a serum containing medium was an acquired property of these cells. In serum free medium, both monocytes and lymphocytes adhered but detachment of the monocytes did not permit adequate recovery. The R/O monocytes suspended in serum did not adhere readily to plastic but did so to microexudate-coated plates.

Gelatin-coated plates incubated with plasma absorb the cell adherence-promoting protein fibronectin (19,67). When added to such plates, monocytes of the 24A fraction demonstrated relatively low adherence at all incubation times up to 8 hours. In contrast, a moderate number of 24B monocytes and nearly all 28 ml/minute monocytes adhered after 1 hour although longer incubation times caused the majority of monocytes to detach (Table 6). R/O monocytes adhered to fibronectin-coated plates readily and so strongly that they could not be detached.

Nylon wool columns often are used to separate lymphocytes and NK cells from monocytes using a 1 hour incubation in a serum containing medium (81). Under such conditions, however, only 18% of the 24A monocytes and 40% of the 24B monocytes adhered although the 28 ml/minute and R/O monocytes were adherent (74 and 77% respectively). After 4 hours incubation on the column, greater than 70% of the 24 ml/minute monocytes were retained.

#### Percoll Gradient Separation

Cells isolated by CCE were subfractionated by density using a continuous Percoll gradient (Table 7). Cells of the 24A fraction formed one diffuse and two distinct bands. One distinct band corresponded to a mean density of 1.062 g/ml and contained 85% of the Kurloff cells applied to the gradient with a purity of 91%. Kurloff cells had a single volume peak at  $230\mu^3$ . A second distinct band was identified at a mean density of 1.075 g/ml which contained 86% of the applied monocytes with a purity of 35%. This band which also contained 65% lymphocytes, had a bimodal volume peak with maximums at  $153\mu^3$  (lymphocytes) and  $283\mu^3$  (monocytes). The morphology of the Kurloff cells and small monocytes isolated from the 24A fraction by Percoll gradient centrifugation is presented in Figures 9 and 10. Cells of the 24B fraction yielded the same two density bands as the 24A fraction. The 1.062 g/ml band had a Kurloff cell purity of 60% ( $247\mu^3$ ) while the 1.075 g/ml

TABLE 6. ADHERENCE CHARACTERISTICS OF GUINEA PIG MONOCYTES.

Conditions	% Adherence of Monocytes Contained In				
	Post Ficol-Hypaque Cells	Small Mononuclear Cell Fraction 24A	Large Monocyte Fraction 24B (28 ml/min)	R/O Monocyte Fraction	
Plastic Culture Flasks					
4 hours	2	2	1	0	17
Micro-exudate Flasks					
1 hour	NT	0	0	0	29
4 hours	NT	6	15	26	74
Gelatin-Fibronectin Plates <sup>a</sup>					
1 hour	73	19	55	93	91
4 hours	68	20	NT	38	99
8 hours	71	37	30	33	NT
Nylon Wool Columns					
1 hour	54	18	40	74	77
4 hours	98	71	75	93	98
8 hours	100	60	90	99	100

<sup>a</sup>Data were acquired using guinea pig plasma as the source of fibronectin. Essentially similar results were obtained using purified human fibronectin.

Mean data from 3 experiments.

NT = not tested

TABLE 7. MONOCYTE ENRICHMENT AND ISOLATION OF KURLOFF CELLS FROM CCE FRACTIONS USING PERCOLL DENSITY GRADIENTS.

Determination	POST-ELUTRIATION FRACTIONS <sup>a</sup>					
	24 A		24B		28 ml/min	
	Kurloff	Monocytes	Kurloff	Monocytes	Kurloff	Monocytes
Numbers of cells x 10 <sup>5</sup>	48	78	4.8	41	8.1	89
Percent composition <sup>b</sup>						
OCE fractions	3	5	10	45	7	81
Percoll fractions						
1.062 g/ml	91	0	60	2	78	2
1.075 g/ml	0	35	8	85	13	85
Mean volume ( $\mu^3$ )	230	283	247	300	300	317

a. Counter flow centrifugation elutriation (CCE) was performed at constant rotor speed of 3,000 RPM and collections made at 2 flow rates. The 24 ml/minute collection was made in a total volume of 400 ml. The first 200 ml fraction was designated 24A and the second 200 ml fraction as 24B. The 28 ml/minute collection was made in a total volume of 200 ml.

b. Cell percentages are reported for monocytes and Kurloff cells with the remaining cells in each fraction being lymphocytes. The majority of lymphocytes formed a diffuse band at a density of 1.080.

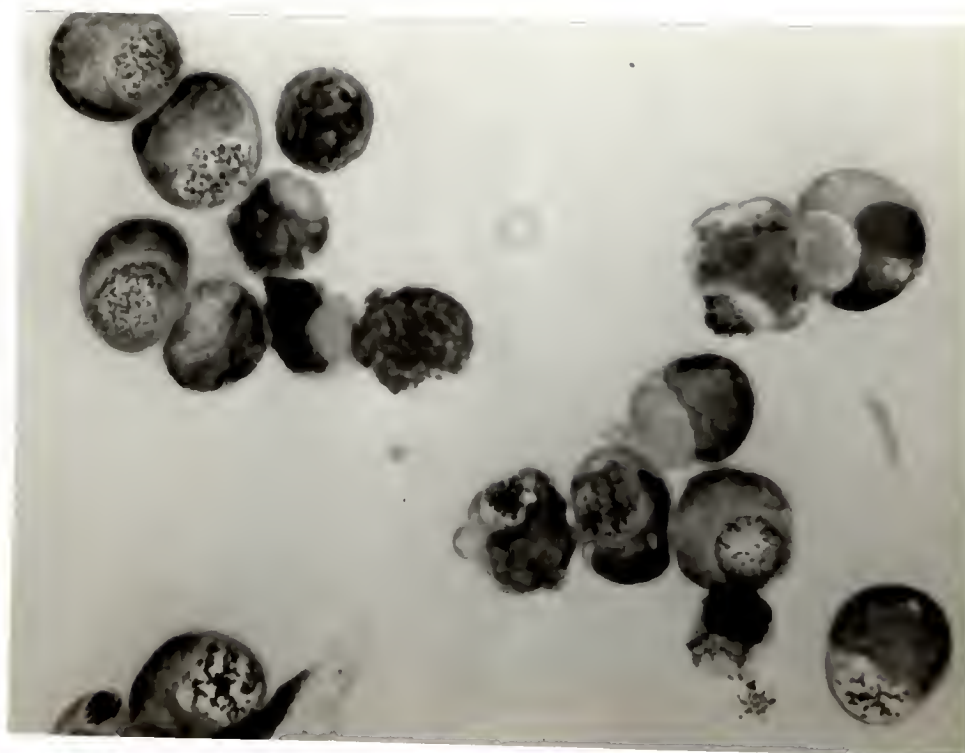


FIGURE 9. Morphology of Kurloff cells and small monocytes of the 24A fraction after Percoll density gradient centrifugation (x 2500). Mean density of 1.062 g/ml. This band is composed of 91% Kurloff cells. Note the eccentric inclusion bodies which characterize these cells.

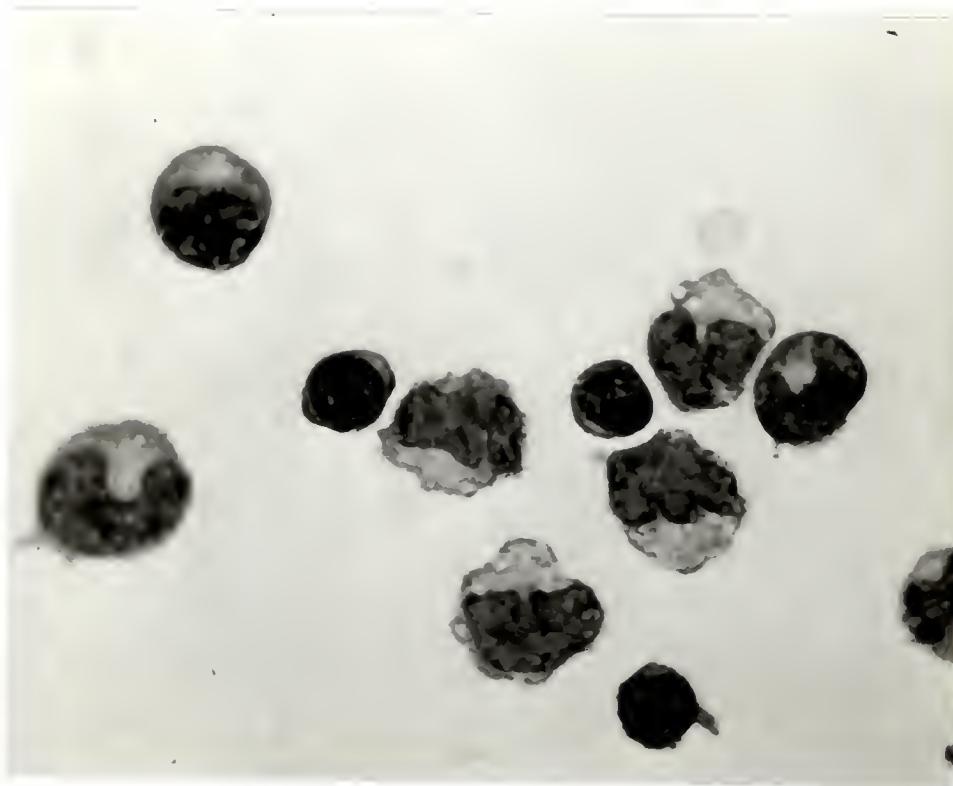


FIGURE 10. Morphology of Kurloff cells and small monocytes of the 24A fraction after Percoll density gradient centrifugation (x 2500). Mean density of 1.075 g/ml. This band is enriched in small monocytes (35%) although lymphocytes are still the predominant cell type.

band had a monocyte concentration of 85% ( $300\mu^3$ ) with an 80% monocyte recovery. The majority of lymphocytes from both fractions formed a diffuse band below a density of 1.080 g/ml. Percoll gradient separation of the 28 ml/minute fraction yielded one distinct band at a mean density of 1.075 g/ml. This band was composed of 85% monocytes (70% yield) and had a single volume peak identical to that of the original preparation ( $317\mu^3$ ).

It was concluded that a combination of CCE and Percoll gradient separation resulted in a method to isolate (a) Kurloff cells in high yield and purity, (b) a population of small monocytes ( $283\mu^3$ ) with a purity of 35% enriched from 5% of the 24A fraction and (c) a population of intermediate-sized monocytes ( $300\mu^3$ ) of high purity (85%) from the 24B fraction. While Percoll gradient separation did not improve the purity of the large monocytes isolated by CCE at 28 ml/minute, it did demonstrate that these monocytes had a mean density similar to that of small and intermediate-sized monocytes.

### Phagocytosis

#### A. Antibody-Independent Phagocytosis

Non-immune phagocytosis was performed using two substrates: carbon particles and polystyrene latex spheres. Both particles are frequently used to demonstrate phagocytosis and are readily engulfed by macrophages of a variety of animal species including guinea pig.

As shown in Table 8, virtually no carbon was ingested by 24A, 24B or 28 ml/minute monocytes within a 4 hour time period. In contrast, R/O monocytes readily engulfed this material within 30 minutes. The amount of carbon ingested by R/O monocytes did not increase from 30 minutes to 120 minutes but almost doubled over the ensuing 2 hours of incubation. Post-Ficoll-Hypaque monocytes demonstrated a very low amount of carbon ingestion consistent with the small numbers of R/O monocytes found in this preparation.

Phagocytosis of albumin-coated fluorescent polystyrene spheres (Covaspheres<sup>R</sup>) was next examined using both cytocentrifuge and wet-mount preparations under a Zeiss epifluorescent microscope. After 15 minutes of incubation, monocytes of the 24A, 24B and 28 ml/minute fractions had beads bound to their surfaces but very few beads had been ingested (Table 9). Concurrently, the R/O monocytes had very few surface bound beads but did contain numerous intracellular beads demonstrating that these cells were avidly phagocytic. By 60 minutes of incubation, surface bound beads appeared to have been ingested, as beads no longer were attached to the cell surface of any monocyte fraction (Figures 11-13). Monocytes from the 24A fraction had the lowest phagocytic activity while the 24B and 28 ml/minute monocytes showed moderate bead ingestion. The R/O monocytes had the highest phagocytic activity, although the number of engulfed beads had not appreciably changed between 15 and 60 minute periods. A few R/O monocytes had

ingested so many beads that accurate counting was not always possible and some appeared to have ruptured. Examination of the phagocytic index revealed that fewer monocytes of the 24A fraction were phagocytic than in the other monocyte fractions. Since the avidity index indicated that approximately the same number of beads were ingested by cells actually engaged in phagocytosis in all fractions except the R/O cells, the low phagocytic activity in the 24A fraction appeared to be due to fewer monocytes actually engaging in phagocytosis.

#### B. Antibody-Dependent Phagocytosis

Both elutriated and unfractionated monocytes were evaluated for differences in their ability to phagocytize sheep erythrocytes opsonized with rabbit anti-SRBC IgG. After thirty minutes, only 28 ml/minute and R/O monocytes demonstrated a moderate degree of phagocytosis (Table 10). The small monocytes of the 24A fraction showed a low rate of engulfment even after 4 hours of incubation whereas the 24B monocytes were moderately phagocytic after 2 hours. The monocytes of the 28 ml/minute and R/O fractions displayed a continual increase in the phagocytic rate with increased time. After 4 hours, it was difficult to count the erythrocytes in some R/O monocytes due to the large quantities ingested as well as their resultant fusion within the phagocytic vacuoles. The phagocytic index revealed a gradation in the percentage of monocytes which

were phagocytic with the 24A monocytes having the lowest percentage and the R/O having the greatest number of actual phagocytes. In contrast to the phagocytic avidity for latex beads, a difference in phagocytic avidity was observed between the monocyte fractions. Monocytes of the 24A fraction had low phagocytic avidity, monocytes of the 24B and 28 ml/minute fractions intermediate avidity, and R/O monocytes high phagocytic avidity for opsonized erythrocytes. Accordingly, the very low phagocytic activity of the 24A fraction for opsonized erythrocytes was due to low phagocytic avidity as well as very few monocytes actually engaging in phagocytosis.

#### C. C3-Dependent Phagocytosis

Whole cells and cell wall components derived from Candida albicans are known to activate the alternative complement pathway. Further, Morrison and Cutler (144) have shown that phagocytosis of this fungus by murine macrophages is dependent upon C3 but not upon antibody specific for Candida albicans.

We first determined whether or not phagocytosis of Candida albicans by guinea pig monocytes was similar to murine macrophages in being dependent upon a heat labile serum opsonin. Figure 14 presents the kinetics of yeast uptake by post-Ficoll-Hypaque monocytes in the absence of serum or in the presence of fresh serum or serum inactivated by heating at 56°C for 60 minutes. Over a 4

hour time period, phagocytosis of Candida albicans in the presence of heat inactivated serum was identical to the uptake of the yeast in the absence of serum. These results demonstrated not only that there was no heat stabile opsonin in guinea pig serum which supported Candida albicans phagocytosis but also that fresh guinea pig serum contained a heat labile opsonin that markedly increased phagocytosis within 60 minutes although some organisms continued to be engulfed over the ensuing 2 hours. This decreased rate of uptake after 60 minutes suggested that the monocytes were reaching their maximum phagocytic capacity. The dramatic increase in ingestion in the presence of fresh serum compared to heat inactivated serum supports the contention of Morrison and Cutler (144) that a heat labile opsonin, presumably C3, is involved in the phagocytosis of Candida albicans. When Candida albicans was opsonized with fresh guinea pig sera rendered C3 deficient by incubation with anti-C3, less than 2 organisms/100 phagocytes were ingested by post-Ficoll-Hypaque monocytes in 60 minutes. Thus C3 must play an integral role in the phagocytosis of Candida albicans by guinea pig monocytes.

Having demonstrated that a heat labile factor in serum promotes phagocytosis of Candida albicans by post-Ficoll-Hypaque guinea pig monocytes, the next experiment examined the complement dependent phagocytosis of the monocyte fractions isolated by CCE. As observed in Table 11, all

monocyte fractions were similar to the post-Ficoll-Hypaque monocytes in that each monocyte fraction required a heat labile component of serum for optimal phagocytosis of Candida albicans. While heat inactivated serum promoted a low rate of phagocytosis, opsonized yeast were ingested readily by all monocyte fractions. However, phagocytosis by the 24A monocytes appeared to take longer than that of the R/O or 28 ml/minute monocytes. After 120 minutes, however, the 24A monocytes had ingested only 27% fewer yeast than the 28 ml/minute monocytes. This modest reduction in phagocytic activity was due entirely to a reduced percentage of active phagocytes and not to a reduced phagocytic avidity. Thus, ingestion of Candida albicans in the presence of a heat labile opsonin was similar to non-immune phagocytosis of polystyrene beads in that both particles were readily ingested by all 4 monocyte fractions. The modest decrease in phagocytic activity by monocytes of the 24A fraction was due entirely to a decrease in phagocytic index rather than a decrease in phagocytic avidity. In contrast, the antibody-dependent phagocytosis of opsonized erythrocytes and the non-immune ingestion of carbon particles was markedly reduced in the 24A fraction compared to the other monocyte fractions. This reduced phagocytic activity was due not only to a reduced percentage of active phagocytes but also to a reduced phagocytic avidity for these particles.

TABLE 8. PHAGOCYTOSIS OF CARBON PARTICLES BY GUINEA PIG MONOCYTES ISOLATED BY CCE.

Monocyte Fraction	Phagocytic Activity <sup>a</sup> Relative Carbon Uptake/100 Monocytes/ Minutes Indicated				Phagocytic Index <sup>b</sup>
	60	120	240		
Post Ficoll-Hypaque	2.0	3.0	5.0		2
24A	0	0	0		0
24B	0	1.0	1.0		1
28 ml/minute	0	1.0	2.0		2
R/O	120	130	230		62

a. The amount of carbon ingested was scored on a 0 to 4+ scale of intensity. The value reported is the cumulative score for 100 monocytes.

b. This figure represents the percentage of monocytes in each fraction which displayed definite carbon phagocytosis as graded equal to or greater than 1+ after 240 minutes of incubation.

TABLE 9. PHAGOCYTOSIS OF ALBUMIN-COATED, FLUORESCENT POLYSTYRENE BEADS BY GUINEA PIG MONOCYTES ISOLATED BY OCE.

Monocyte Fraction	Phagocytic Activity		Phagocytic Index <sup>b</sup>	Avidity Index <sup>c</sup>
	Engulfed Beads/100 Monocytes <sup>a</sup> 15 Minutes	60 Minutes		
Post Ficoll-Hypaque	380 ± 18	840 ± 28	86	9.8
24A	100 ± 6	280 ± 27	60	4.7
24B	150 ± 23	360 ± 27	71	5.1
28 ml/minute	180 ± 18	390 ± 23	76	5.1
R/O	1170 ± 91	1150 ± 38	88	13.1

a. The results represent the mean ± S.E. of 4 experiments.

b. The phagocytic index is the percentage of monocytes actually ingesting beads at 60 minutes.

c. The avidity index is the mean number of beads in cells engaged in phagocytosis at 60 minutes.

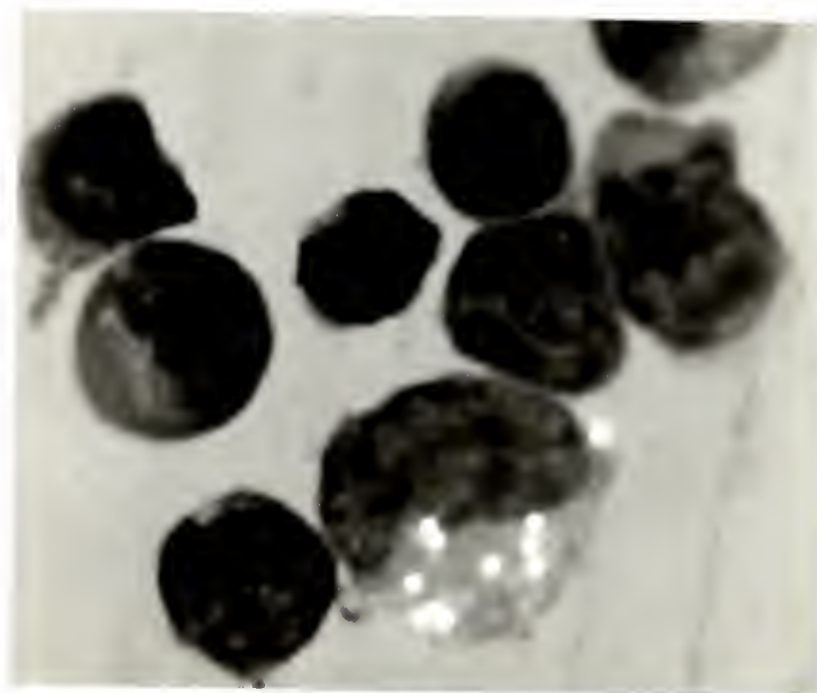


FIGURE 11.

Phagocytosis of albumin-coated fluorescent polystyrene beads by guinea pig monocytes isolated by CCE. (Photographed by using epifluorescence microscopy x 2500). The 24A fraction (24 ml/minute, 0-200 ml collection) after incubation for 60 minutes at 37°C. 60% of small monocytes ingest a moderate number of beads whereas the remainder of the monocytes are not phagocytic.

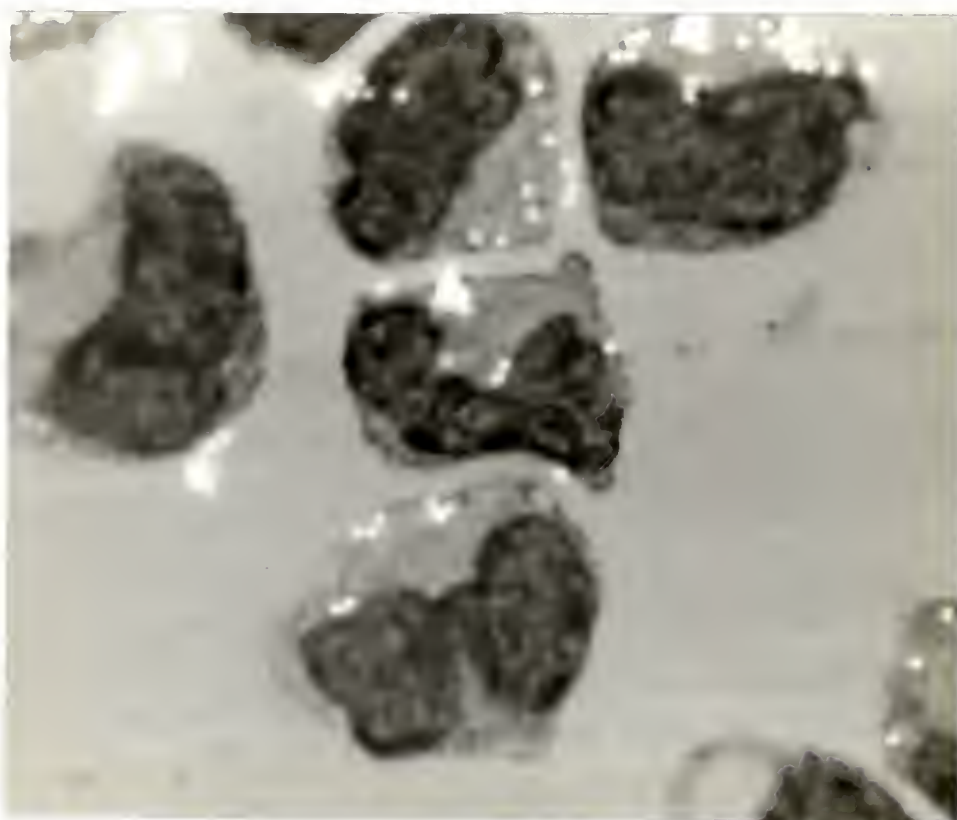


FIGURE 12.

Phagocytosis of albumin-coated fluorescent polystyrene beads by guinea pig monocytes isolated by CCE. (Photographed using epifluorescence microscopy x 2500). 28 ml/min fraction after incubation for 60 minutes at 37°C. A greater percentage of intermediate-sized monocytes (76%) are phagocytic compared to small monocytes (60%).

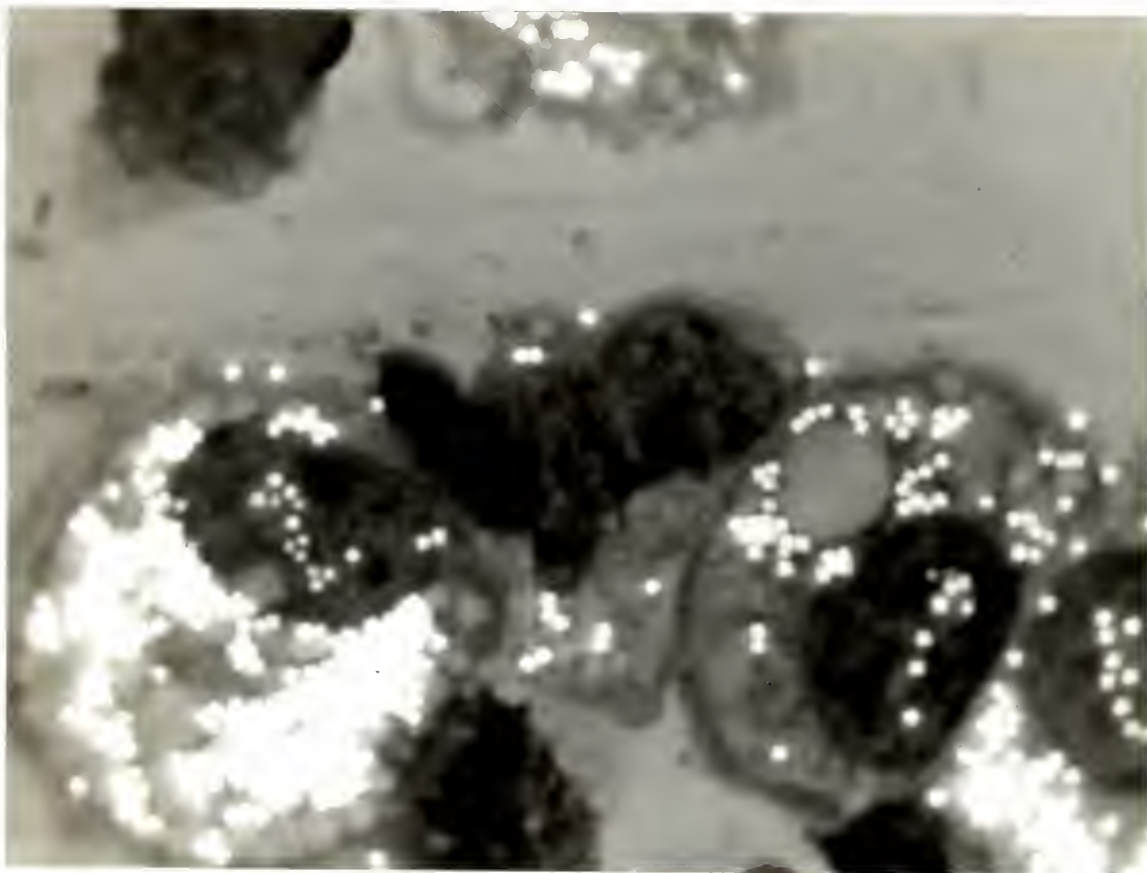


FIGURE 13.

Phagocytosis of albumin-coated fluorescent polystyrene beads by guinea pig monocytes isolated by CCE. (Photographed using epifluorescence microscopy x 2500). The rotor-off (R/O) fraction after incubation for 60 minutes at 37°C. This fraction contains the highest percentage of phagocytic monocytes (88%). Unlike the smaller monocyte fractions, these cells demonstrate greater avidity for the albumin-coated beads as shown in this photomicrograph.

TABLE 10. PHAGOCYTOSIS OF ANTIBODY-COATED SHEEP ERYTHROCYTES BY GUINEA PIG MONOCYTES ISOLATED BY OCE.

Monocyte Fraction	Erythrocytes 30'	Phagocytic Activity				Phagocytic Index <sup>a</sup>	Avidity Index <sup>b</sup>
		Ingested/100 Monocytes	60'	120'	Minutes Indicated 240'		
Post Ficoll-Hypaque	20 ± 4	37 ± 5	64 ± 11	161 ± 27	40	4.0	
24A	19 ± 9	25 ± 8	21 ± 5	37 ± 15	19	1.9	
24B	23 ± 10	55 ± 24	53 ± 11	117 ± 26	30	3.9	
28 ml/minute	26 ± 7	69 ± 13	114 ± 12	170 ± 12	55	3.1	
R/O	98 ± 31	271 ± 71	392 ± 78	527 ± 62	79	6.7	

The sheep erythrocytes were opsonized with rabbit anti-sheep red blood cell immunoglobulin and incubated at a 100:1 erythrocyte to monocyte ratio. The results represent the mean ± S.E. of 4 experiments.

- a. The phagocytic index is the percentage of monocytes actually ingesting opsonized erythrocytes at 240 minutes.
- b. The avidity index is the mean number of erythrocytes in cells engaged in phagocytosis at 240 minutes.

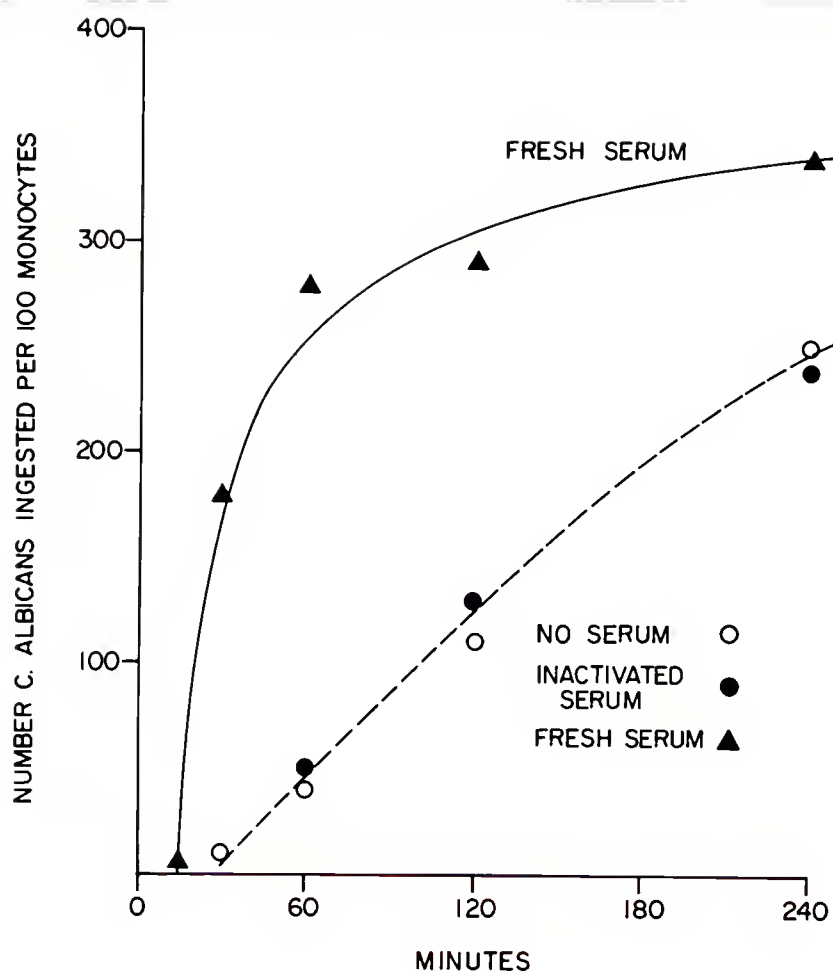


FIGURE 14.

Kinetics of yeast uptake by guinea pig post-Ficoll-Hypaque monocytes demonstrating enhanced phagocytosis in the presence of a heat labile opsonin. *Candida albicans* was incubated in medium alone, fresh guinea pig serum or heat inactivated serum (56°C, 30 minutes) for 15 minutes at 4°C at a 10:1 ratio of organisms to phagocytes. Wright-Giemsa stained preparations were used to evaluate the number of yeast ingested per 100 monocytes.

TABLE 11. PHAGOCYTOSIS BY GUINEA PIG MONOCYTES OF CANDIDA ALBICANS OPSONIZED BY FRESH GUINEA PIG SERUM.

Monocyte Fraction	Phagocytic Activity					
	Yeast Ingested/100 Monocytes/Minutes Indicated					
	Heat		Inactivated		Opsonized	
	Serum	60	Serum	60	Fresh Serum	120
					30	
Post Ficoll-Hypaque	50		180	280	290	85
24A	10		40	190	220	60
24B	14		100	230	290	95
28 ml/minute	30		180	260	350	91
R/O	65		270	260	310	88
						3.4
						3.7
						3.1
						3.8
						3.5

a. The phagocytic index is the percentage of monocytes actually ingesting Candida albicans at 120 minutes.

b. The avidity index is the mean number of Candida albicans in cells engaged in phagocytosis at 120 minutes. Data are reported as the mean of 3 experiments.

### Fc Receptors

Guinea pig monocytes were evaluated for the presence of Fc receptors by a rosette assay in which three sources of red blood cells were used. The results are summarized in Table 12.

In the first assay, sheep red blood cells were sensitized with either rabbit or autologous guinea pig anti-sheep erythrocyte immunoglobulin. Although the customary assay for Fc receptors on monocytes uses rabbit anti-sheep immunoglobulin (180), autologous antisera was also investigated to determine if more rosettes would form when both Fc receptor and antibody were derived from the same species. In general, the percentage of monocytes forming rosettes with autologous antisera was equivalent to or slightly greater than the number obtained with rabbit anti-sheep immunoglobulin. Using sheep erythrocytes sensitized with rabbit anti-sheep immunoglobulin, the post-Ficoll-Hypaque monocytes were 35% Fc receptor positive when examined on cytocentrifuge slides, while direct analysis of the cells in suspension on glass slides under coverslips yielded 45% Fc receptor positive monocytes. Since this difference was consistently observed for all monocyte preparations examined regardless of the indicator erythrocyte or source of antibody, the results of the two methods of observation were averaged. Thus, post-Ficoll-Hypaque monocytes averaged 40% Fc receptor positive monocytes. Monocytes of the 24A fraction were 99% Fc

receptor positive, the 24B monocytes 35% and the 28 ml/minute monocytes only 12%. The Fc receptor positive monocytes of these fractions bound an average of 3.5 erythrocytes per monocyte. The R/O monocytes were 85% Fc receptor positive and averaged greater than 10 erythrocytes per rosette. Other Fc receptor positive cells in the 24A fraction (average of 3 erythrocytes/cell) were identified as lymphocytes (presumably B lymphocytes). These non-monocyte Fc receptor positive cells usually comprised 5 to 7% of the 24A fraction. In the 24B and 28 ml/minute fractions, Fc receptor positive lymphocytes constituted 2% of the cells of each fraction. A variable number of Fc receptor positive cells in the R/O fraction were identified as lymphocytes (2-10%) and granulocytes (8-15%).

Monocytes of the 24A and 24B fractions did not ingest sheep erythrocytes opsonized with rabbit immunoglobulin during the 30 minute incubation period used to determine Fc receptors. During this same interval, 12% of the monocytes of the 28 ml/minute fraction ingested erythrocytes for a total of 28 erythrocytes/100 monocytes (phagocytic avidity of 2.3 erythrocytes/phagocyte). The lower rate of ingestion of sensitized erythrocytes observed with the Fc receptor assay compared to the phagocytosis assay (Table 10) at comparable incubation times was accounted for by the difference in the ratio of erythrocytes to monocytes used (phagocytosis assay 100:1; Fc receptor assay 10:1).

In the second assay, the source of erythrocytes was varied and erythrocytes from ox (bovine) and human were used. Sensitized ox erythrocytes usually are more reactive with weak Fc receptors. However, this did not prove to be the case for guinea pig monocytes. Sensitized ox erythrocytes consistently revealed fewer Fc receptor positive monocytes than did sheep erythrocytes sensitized with either rabbit or guinea pig anti-sheep immunoglobulin (Table 12). This was most dramatically revealed with the 24A fraction where 99% of the monocytes reacted with sensitized sheep erythrocytes whereas only 19% reacted with sensitized ox erythrocytes. Human erythrocytes sensitized with human anti Rh<sub>0</sub>D immunoglobulin were even less reactive than sensitized ox erythrocytes. Only 1 to 2% of the monocytes in the post-Ficoll Hypaque preparation formed rosettes with sensitized human erythrocytes and no Fc receptor positive monocytes were found in the 24A, 24B or 28 ml/minute fractions. However, the R/O fraction contained 20% Fc receptor positive monocytes which averaged 5 human red blood cells per rosetting monocyte.

#### Development of Fc Receptors in Culture

In a previous study of guinea pig monocyte Fc receptors, Brade and co-workers reported that nearly all monocytes expressed Fc receptors (23). However, their study was performed after placing post-Ficoll-Hypaque monocytes in culture for 48 hours. Since current results

showed only 40% of post-Ficoll-Hypaque monocytes possessed Fc receptors when tested immediately after isolation, it was hypothesized that monocytes might be induced to express Fc receptors in culture.

Post-Ficoll-Hypaque monocytes and monocytes isolated by CCE were tested for Fc receptors immediately after isolation and after 48 hours of culture. Teflon bottles were used for culture to prevent adherence and to allow recovery of all monocytes. Analysis for Fc receptors was performed using sheep erythrocytes sensitized with rabbit anti-sheep erythrocyte immunoglobulin. As seen in Table 13, expression of Fc receptors on post-Ficoll Hypaque monocytes increased from 41% at two hours after separation to 97% after 48 hours of culture. Examination of the individual CCE fractions revealed that 24A monocytes remained Fc receptor positive in culture while the 28 ml/minute fraction increased from 12% to 98% Fc receptor positive. In fact, 70% of 28 ml/min monocytes were Fc receptor positive after 4 hours in culture. Rosette formation was completely blocked by the addition of 100 mM of 2-deoxyglucose suggesting that the expression of Fc receptors was an energy dependent process. Similarly, Fc receptor expression increased in the 24B fraction and the R/O monocytes so that Fc receptors expression on these cells averaged 81% and 99% respectively.

TABLE 12. Fc RECEPTOR ANALYSIS OF GUINEA PIG MONOCYTES SEPARATED BY OCE.

Determinations	Monocyte Fc Receptors					
Assay conditions						
Source of erythrocytes	sheep	sheep	ox			human
Source of anti-erythrocyte IgG	rabbit	G. pig	rabbit			human
Number of assays	6	4	3			6
Percentage of rosetting monocytes <sup>a</sup>						
Post Ficoll-Hypaque	40 ± 2	45 ± 2	11 ± 2			2 ± 1
24A	99 ± 1	99 ± 1	19 ± 2			0.3 ± 0.2
24B	36 ± 1	49 ± 2	14 ± 2			0.2 ± 0.2
28 ml/minute	12 ± 1	15 ± 2	5 ± 1			0.2 ± 0.2
R/O	85 ± 2	95 ± 2	40 ± 3			20 ± 1

a. The results obtained using cytocentrifuge slides and direct phase-contrast microscopic examination were averaged to determine the percent of monocytes forming rosettes with sensitized erythrocytes in a 30 minute incubation at 37°C at a 10:1 erythrocyte to monocyte ratio. The results are reported as the mean ± S.E.

TABLE 13. EXPRESSION OF Fc RECEPTORS IN CULTURED GUINEA PIG MONOCYTES.

Monocyte Fraction	Percentage of Fc Receptor Positive Monocytes <sup>a</sup>	
	2 Hours After Isolation	48 Hours After Isolation
Post Ficoll-Hypaque	41 ± 2	97 ± 2
24A	98 ± 2	96 ± 1
24B	34 ± 2	81 ± 2
28 ml/minute	12 ± 2	98 ± 2
R/O	85 ± 3	99 ± 1

a. Results were obtained using sheep erythrocytes sensitized with rabbit anti-sheep erythrocyte IgG at a 10:1 ratio of RBC to monocytes. The dilution of incubation was 30 minutes. Data are reported as mean ± SE of 3 experiments.

### Antibody-Dependent Cellular Cytotoxicity

The ability of guinea pig monocytes to participate in antibody-dependent cellular cytotoxicity (ADCC) was investigated using chromium labeled sheep or human erythrocytes as target cells. Sheep erythrocytes when used as target cells were sensitized with either rabbit or autologous guinea pig anti-sheep erythrocyte IgG. Human erythrocytes were sensitized with human anti-Rh<sub>0</sub>D IgG. The ADCC assay was performed at an effector to target ratio of 1:1 or 10:1 and reported as the percent specific isotope released from lysed red cells after 8 or 16 hours of incubation.

As seen in Table 14, the post-Ficoll-Hypaque monocytes displayed a modest degree of ADCC against both sheep and human erythrocyte targets with specific isotope release increasing as the duration of incubation increased. Increasing the effector to target cell ratio did not alter the lysis of sheep erythrocytes sensitized with rabbit antibody but did so when these cells were sensitized with autologous guinea pig antibody. The 24A and 24B monocytes displayed less ADCC activity than the 28 ml/minute fraction or the R/O cells. For all monocyte fractions examined, the maximum ADCC activity was observed with autologous guinea pig antibody directed against sheep erythrocytes.

The above test of ADCC activity did not correlate with the presence of Fc receptors since the 24A and 24B fractions had very low levels of ADCC activity although 99%

of the monocytes of the 24A fraction and 49% of the monocytes of the 24B fraction displayed Fc receptors (Table 12) when measured using sheep RBC sensitized with guinea pig antisera. This dicotomy might arise if the presence of lymphocytes within these two fractions inhibited monocyte ADCC activity by steric hindrance as has been demonstrated for human small monocytes (128). This possibility was explored in the following two experiments.

In the first experiment, Percoll density gradients were used to enrich the effector cell population. This experiment was conducted on the assumption that if lymphocytes suppressed the ADCC activity of monocytes of the 24A fraction, then the percent specific isotope release from chromium labeled target cells should increase as the purity of the monocytes increased from 5% in the 24A CCE fraction to 35% in the 1.075 g/ml band on Percoll. Despite this purification, however, the Percoll purified monocytes did not demonstrate significant ADCC activity nor was this low level of activity increased over that observed in the whole 24A fraction (Table 15). Additionally, purification of 24A monocytes on Percoll did not alter Fc receptors since after separation 95% of the monocytes formed rosettes with rabbit anti-sheep erythrocytes. Similarly, monocytes of the 24B fraction were enriched by Percoll gradient centrifugation to 85% purity and these more purified monocytes did exhibit more ADCC activity over that of the unfractionated preparation (20% specific isotope release vs

5%). Using Percoll gradients and the cells of the 24A fraction, it was possible also to examine the ADCC activity of highly purified Kurloff cells and the lymphocytes contained in this fraction. Kurloff cells localized at a mean density of 1.062 g/ml on Percoll and were recovered at a purity of 91%. When tested at a 1:1 or 10:1 effector to target cell ratio, these cells demonstrated no ADCC activity (Table 15). Similarly, the lymphocytes recovered at a mean density of 1.080 g/ml in greater than 95% purity did not demonstrate ADCC activity. Consistent with their absence of ADCC activity, the Kurloff cells did not form rosettes with antibody-coated sheep erythrocytes implying a lack or low density of Fc receptors which presumably are necessary for participation in ADCC. Further, only 5% of lymphocytes contained within the 1.080 g/ml fraction possessed Fc receptors. All Percoll cell fractions examined had viabilities greater than 95%.

In the second experiment, the question was addressed as to whether or not the presence of lymphocytes would suppress monocyte ADCC activity. The 24A lymphocytes obtained from the 1.080 band of Percoll were added to 28 ml/minute monocytes and ADCC activity determined at a monocyte to target ratio of 1:1 (Table 6). The addition of an equal number of lymphocytes to the 28 ml/min monocytes caused significant suppression of ADCC. Complete ablation of ADCC activity was demonstrated when lymphocytes composed 65% of the effector cell population. Thus, the high

TABLE 14. ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY (ADCC) ACTIVITY OF GUINEA PIG MONOCYTES SEPARATED BY CCE.

Cr <sup>51</sup> Labeled Target Cells	Target:Effector Ratio	Duration Incubation (hours)	Post Ficoll- Hypaque	Percent Specific Isotope Release with Effector Monocyte Fractions				R/O
				24A	24B	28		
Sheep erythrocytes rabbit anti-SRBC	1:1	8	9 ± 1	2 ± 0.3	6 ± 1	16 ± 1		23 ± 1
		16	20 ± 1	4 ± 1	6 ± 1	23 ± 2		27 ± 2
	10:1	8	6 ± 1	1 ± 0.4	4 ± 0.5	14 ± 1		NT
		16	16 ± 1	8 ± 1	7 ± 1	20 ± 1		NT
guinea pig anti-SRBC	1:1	16	11 ± 2	6 ± 1	8 ± 1	23 ± 1		17 ± 2
	10:1	16	20 ± 2	9 ± 0.5	9 ± 1	22 ± 1		NT
Human erythrocytes human anti-Rh <sub>0</sub> D IgG	1:1	8	3 ± 1	1 ± 0.4	2 ± 0.4	8 ± 1		14 ± 2
		16	11 ± 1	1 ± 0.4	2 ± 1	12 ± 1		19 ± 1
	10:1	8	11 ± 1	1 ± 0.3	3 ± 1	14 ± 1		13 ± 1
		16	9 ± 1	2 ± 1	2 ± 0.4	11 ± 2		22 ± 2

Results represent the mean ± S.E. of 6 experiments with rabbit anti-sheep RBC and 4 experiments each for guinea pig anti-sheep RBC and human anti-Rh<sub>0</sub>D IgG. Spontaneous release averaged 1.3%/hr for sensitized sheep and human erythrocytes respectively.

NT = not tested.

TABLE 15. ADOC ACTIVITY OF PERCOLL PURIFIED OCE SEPARATED MONOCYTES

Effector Cell Fraction	Effector Cell Tested and Differential Percentages <sup>a</sup>	% Specific Isotope Release at Effector to Target Ratios 1:1b    1:1c    10:1b		
24A OCE Fraction	Monocyte	(5%)	4	5    8
Percoll fractions				
1.062 g/ml	Kurloff cell	(91%)	1	1
1.075 g/ml	Monocyte	(35%)	4	6    4
1.080 g/ml	Lymphocyte	(100%)	2	2    1
24B OCE Fraction	Monocyte	(64%)	6	5    NT
Percoll fraction				
1.075 g/ml	Monocyte	(85%)	22	20    NT

a. Results of two experiments using sheep erythrocytes sensitized with rabbit anti-sheep RBC IgG in a 16 hour assay.

b. Effector:target cell ratio based on total cell number.

c. Effector:target cell ratio based on the effector cell type tested.

TABLE 16. SUPPRESSION OF ADCC IN THE 28 ML/MIN FRACTION BY THE ADDITION OF LYMPHOCYTES.

Number of Monocytes in Assay	Number of Lymphocytes Added to Assay	Percentage of Lymphocytes in Assay	Percent Specific Isotope Release	Percent Inhibition of ADCC
$2 \times 10^5$	0	8	22	0
$2 \times 10^5$	$2 \times 10^5$	50	14	36
$2 \times 10^5$	$4 \times 10^5$	65	1	99.5

All assays were determined after 16 hours of incubation using a 1:1 ratio of monocytes to sheep erythrocytes sensitized with rabbit anti-sheep RBC IgG. Results are the mean of 2 experiments.

lymphocyte percentage of the 24A fraction may be preventing the small monocytes from mediating ADCC.

### Tumoricidal Activity

Guinea pig mononuclear cells were tested for tumoricidal activity using  $^{125}\text{I}$ UDR labeled P-815 mastocytoma cells as tumor targets. The objective of the first experiment was to determine if post-Ficoll-Hypaque mononuclear cells were tumoricidal and if so to determine the optimal time and ratio of effector to target cells for tumoricidal measurements. Specific isotope release from target cells was negligible at both the 4 and 8 hour assays at all effector to target cell ratios tested (Table 17). However, appreciable tumoricidal activity was observed after 16 hours at effector to target ratios equal to or exceeding 10:1. Ratios higher than 50:1 did not enhance the tumoricidal activity of the mononuclear preparation over that observed at 20:1. From this experiment, we concluded that native, unstimulated tumoricidal activity did exist in the post-Ficoll-Hypaque mononuclear cell preparation of guinea pigs and that an effector to target ratio of either 10:1 or 20:1 was optimal for demonstrating this activity in either a 16 or 48 hour assay.

In the next series of experiments, the individual post elutriation fractions were assayed for tumoricidal activity using the 10:1 effector to target ratio. The level of tumoricidal activity in the 24B and R/O fractions was

similar in magnitude to that of the unfractionated preparation at 16 hours (Table 18). In contrast, high levels of tumoricidal activity were found in the 24A fraction and negligible levels in the 28 ml/minute fraction. In all cases, tumoricidal activity increased when the assay time was lengthened to 48 hours but the results were consistent with those obtained at 16 hours.

Discontinuous Percoll gradients were used to concentrate monocytes and Kurloff cells in an effort to delineate the cell type responsible for the high native tumoricidal activity found in the 24A fraction. It was necessary to use discontinuous gradients of Percoll to purify small monocytes and Kurloff cells instead of continuous gradients in order to obtain the number of cells necessary for the tumoricidal assay. These gradients did not purify Kurloff cells to the same extent as continuous gradients (65% vs. 91%) although monocyte purity was not altered. As shown in Table 19, native tumoricidal activity of the 24A fraction resided in cells isolated from the 1.070 g/ml band of Percoll. This band contained essentially all the monocytes of the 24A fraction although lymphocytes were still the predominant cell type. Equal tumoricidal activity was present in the 1.070 g/ml band whether the effector to target ratio was based on monocytes or lymphocytes. The 1.060 g/ml Percoll band contained sufficient Kurloff cells to test the tumoricidal activity of this cell at a 10:1 effector to target ratio. No

detectable tumoricidal activity was found associated with Kurloff cells.

This series of experiments localized guinea pig native tumoricidal activity to the small sized cells contained in the first CCE collection fraction. Furthermore, Kurloff cells and most lymphocytes contained in this collection did not display tumoricidal activity when tested subsequent to their isolation using discontinuous Percoll gradients. It was concluded that a single monocyte-rich Percoll band at 1.070 g/ml contained essentially all the native tumoricidal activity of guinea pig peripheral blood. However since this fraction contained approximately equal concentrations of small monocytes and lymphocytes, the specific cell type responsible for the tumoricidal activity could not be unequivocally established.

TABLE 17. NATIVE TUMORICIDAL ACTIVITY OF POST FICOLL-HYPAQUE GUINEA PIG MONONUCLEAR CELLS AT VARYING EFFECTOR:TARGET RATIOS OF MONOCYTES TO P-815 MASTOCYTOMA CELLS.

Duration of Incubation (Hours)	% Specific Isotope Release at Effector:Target Ratios <sup>a</sup>				
	1:10	1:1	10:1	20:1	50:1 100:1
4	1 ± 0.4	1 ± 0.4	2 ± 1	2 ± 0.4	5 ± 1 5 ± 0.4
8	2 ± 0.4	3 ± 0.4	3 ± 1	5 ± 1	6 ± 1 6 ±
16	0.5 ± 0.3	3 ± 1	28 ± 3	54 ± 3	62 ± 2 63 ± 3
48	5 ± 1	35 ± 2	64 ± 2	81 ± 3	85 ± 4 65 ± 5

a. Results are reported as the mean ± SE of four experiments. Spontaneous release averaged 1.1%/hr for labeled target cells.

TABLE 18. Native Tumoricidal Activity of Guinea Pig Mononuclear Cells

Monocyte Fraction	Specific Isotope Release <sup>a</sup>	
	Duration of Incubation 16 hours	48 hours
Post Ficoll-Hypaque	22 ± 1	56 ± 4
24A	49 ± 2	64 ± 4
24B	19 ± 1	29 ± 3
28 ml/minute	6 ± 1	11 ± 2
R/O	14 ± 1	27 ± 3

a. All assays were conducted at an effector:target ratio of 10 monocytes per P-815-mastocytoma cell. Data are reported as mean ± SE for 6 experiments at 16 hours and 4 experiments at 48 hours.

TABLE 19. NATIVE TUMORICIDAL ACTIVITY OF THE 24A FRACTION OF GUINEA PIG MONONUCLEAR CELLS BEFORE AND AFTER SEPARATION BY PERCOLL GRADIENT CENTRIFUGATION.

Cell Fraction	Monocyte Concentration %	Specific Isotope Release (16 hours) Effector:Mastocytoma Target Ratio (10:1) based on		
		Monocytes	Lymphocytes <sup>a</sup>	Kurloff Cells <sup>b</sup>
24A OCE	5	49 ± 1	NT	NT
Percoll Gradients				
1.050 g/ml	<1	NT	0.3 ± 0.3	NT
1.060 g/ml	2	15 ± 1	NT	4 ± 0.5
1.070 g/ml	38	47 ± 2	51 ± 2	NT
1.080 g/ml	2	15 ± 2	5 ± 1	NT

a. The lymphocyte concentration was 90% in the 1.050 g/ml fraction, 70% in the 1.070 g/ml fraction and 96% in the 1.080 g/ml fraction.

b. The Kurloff cell concentration was 65% in the 1.060 g/ml fraction but less than 5% in all other fractions isolated by Percoll.

## DISCUSSION

### Separation Procedures for Isolating Guinea Pig Monocytes

Guinea pig monocytes are more dense and smaller in size than human monocytes. Further, the size difference between guinea pig monocytes and lymphocytes is considerably less than the human counterparts (8,151,237). These differences necessitated two important changes in methodology for isolating guinea pig monocytes as compared to human monocytes. First, high density Ficoll-Hypaque and siliconized glass centrifuge tubes were required in order to recover virtually all monocytes from guinea pig peripheral blood and to reduce granulocyte contamination. The latter was undesirable because guinea pig monocytes and granulocytes co-elute during CCE. Siliconized glass, being more hydrophobic than polypropylene, probably decreased granulocyte adherence permitting these cells to centrifuge to the bottom of the tube. Second, relatively high centrifugal force and flow rates were required during CCE. The large diameter difference between human lymphocytes and monocytes allows a clean separation of these two cell populations at low flow rates and centrifugal force (35,62,65,187,237). However, the approximate 2 micron difference in diameter between guinea pig monocytes and

lymphocytes required more centrifugal force and higher flow rates. Volume analysis of Ficoll-Hypaque separated human mononuclear cells reveals two distinct peaks in which lymphocytes ( $187\mu^3$ ) and monocytes ( $407\mu^3$ ) are clearly separate (237), whereas guinea pig monocytes ( $317\mu^3$ ) appear as a shoulder on the size profile of the lymphocytes ( $153\mu^3$ ).

The choice of anticoagulant was critical to good separation of guinea pig monocytes since heparin caused mononuclear cell clumping while ACD and EDTA eliminated all clumping. In addition, it was important to introduce into the elutriation chamber a limited number of cells ( $2 \times 10^8$ ) as satisfactory flow conditions could only be achieved within a narrow cell load range. The choice of eluting to exhaustion at 2 flow rates versus a series of incremental flow rates depended in part on the equipment available. The J-21 Beckman centrifuge cannot maintain precise rotor speed unless its circuitry is altered (213). In preliminary studies not reported here, we found that incremental flow rates were necessary when using this centrifuge to separate guinea pig monocytes. However, the new Beckman J-6B centrifuge has very precise speed control and, when combined with a peristaltic pump capable of providing precisely metered flow, we were able to achieve excellent cell separation by eluting to exhaustion at only two flow rates. By using this system, better separation of small and large cells was achieved than by incremental

increases in flow using either the J-6B centrifuge (Table 3) or the J-21 centrifuge.

Counter-flow centrifugation elutriation consistently separated guinea pig monocytes into three populations differing in both physical and functional properties. Such a separation could not be achieved using Percoll gradients alone because guinea pig monocytes have similar densities and can be isolated within a very narrow density range on a continuous Percoll gradient. Conversely, Percoll gradient separation followed by CCE is not satisfactory because too few cells are recovered from each Percoll band to provide effective separation by CCE. When CCE was followed by Percoll gradients, we were successful in isolating the Kurloff cells in high purity and yield. Further, the technique permitted enrichment of small monocytes contained in the 24A fraction to 35%. As summarized in Table 20, the 24A and 28 ml/minute fractions contained 35% and 40% respectively of peripheral blood monocytes while the 24B fraction contained only 18%. In relation to human monocytes, we believe that the monocytes contained in the 24A fraction correspond to the small monocyte population described for humans while the 28 ml/minute fraction contained monocytes equivalent to human large monocytes. There is no human counterpart so far described to the R/O monocytes of the guinea pig.

Histochemical analysis of CCE separated monocytes revealed heterogeneity in the staining reactions for non-

specific esterase, peroxidase and acid phosphatase. Whereas all guinea pig monocytes contain non-specific esterase, staining intensified with increasing monocyte size. In addition, small monocytes lacked detectable acid phosphatase activity while large monocytes were non-reactive for peroxidase.

Adherence is often used to isolate monocytes and macrophages. However, results reported here clearly indicate that this procedure has serious limitations when used to isolate guinea pig monocytes which may apply to monocytes from other animals as well. Under conditions normally employed for monocyte and macrophage adherence, the majority of guinea pig monocytes were not adherent and the different monocyte fractions isolated by CCE demonstrated profound differences in adherence capacity to a variety of substrates. Therefore, we believe that adherence should not be used to isolate guinea pig monocytes as this procedure inadvertently selects for monocytes which differ in physical and functional properties from the guinea pig monocyte population at large. A similar variance in monocyte adherence may occur in other species as well since monocyte isolation by adherence usually results in a 40% to 60% recovery. Whereas guinea pig small monocytes do not adhere to nylon wool columns under conditions commonly used to remove mononuclear phagocytes from natural killer (NK) cells, this procedure cannot be relied upon to deplete monocytes from

NK cells. This concern is especially valid if the small monocytes of guinea pig are similar to human small monocytes since the latter cells appear to possess natural tumoricidal activity and are more readily activated for cytotoxicity than are large human monocytes.

#### Functional Properties of Guinea Pig Monocytes

Functional characterization of CCE separated guinea pig monocytes showed major differences in phagocytosis, Fc receptors, ADCC and native tumoricidal activity. Counter-flow centrifugation elutriation-separated monocytes displayed marked differences both in the ability to phagocytize different classes of particles and in the rate of ingestion. Although monocytes from the 24A , 24B and 28 ml/minute fractions exhibited similar phagocytic avidity when C3b opsonized Candida albicans and albumin-coated latex spheres were used as substrates, the 24A monocytes had a lower phagocytic index as fewer monocytes in this fraction actually ingested particles. Further, 24A monocytes had both a decreased phagocytic avidity and phagocytic index compared to 28 ml/minute monocytes when tested against antibody-coated erythrocytes. Thus, the 24A fraction had the highest percentage of non-phagocytic monocytes regardless of substrate and certain materials such as carbon were ingested only by monocytes in the R/O fraction. Such data are consistent with the proposition

that guinea pig monocytes are heterogenous with respect to phagocytosis of particulate material.

Heterogeneity in phagocytosis may be due to differences in the type or expression of receptors found on each monocyte subpopulation. Specific receptors have been described for particles opsonized with immunoglobulin (Fc receptors), complement (C3 receptors) and fibronectin (gelatin/denatured protein fibronectin receptors). Alternatively, binding could be mediated by non-specific surface tension forces generated by differences in surface free energy between particles and phagocyte surfaces (222). Although fibronectin binds to gelatinized carbon particles (185) and may bind to albumin-coated latex spheres, it is uncertain whether binding of these particles to the phagocyte is mediated via a fibronectin receptor or non-specific hydrophobic interactions. For instance, binding of fibronectin to bacteria is not sufficient for their opsonization (212) and small monocytes do not bind to a fibronectin-coated surface, suggesting that they may lack the fibronectin receptor. In contrast, the C3b receptor has been found on all three monocyte subpopulations as demonstrated by enhanced uptake of Candida albicans in the presence of C3b.

Less than half of the guinea pig monocytes demonstrated detectable Fc receptors when first isolated. This appeared to reflect the fact that small monocytes expressed the Fc receptor while the intermediate-sized 28

ml/min monocytes did not. It has not been determined whether these latter monocytes truly lack the Fc receptor or if the receptor is of high avidity and is occupied by cytophilic antibody present in serum. The latter case would argue for a difference in receptor avidity or density between the two monocyte populations. Nonetheless, all monocyte fractions were Fc receptor positive after 48 hours in culture. In fact, 70% of the 28 ml/minute monocyte population expressed the receptor after 4 hours in culture. That an energy-dependent step is necessary for this expression is evidenced by the total lack of rosette formation in the presence of 2-deoxyglucose which inhibits glycolysis thus depleting the ATP reserve necessary for rosette formation (139).

Brade et al. and Van Furth described guinea pig monocytes as Fc receptor positive (23,217). However, their Fc receptor assay was performed after 48 hours in culture. In the same study, Van Furth described guinea pig monocytes as loosely adherent when plated on tissue culture surfaces. This observation is consistent with our adherence data and suggests that Van Furth had to wait 48 hours before analyzing the monocytes for Fc receptors in order to obtain a purified preparation of guinea pig monocytes. In contrast, in our experiments we used CCE to rapidly isolate guinea pig monocytes for immediate testing in suspension culture. Such monocytes showed Fc receptor differences which were not apparent after 48 hours in culture.

Two functions mediated by Fc receptors are phagocytosis of IgG opsonized particles, as discussed earlier, and ADCC. Guinea pig monocytes showed increasing ADCC activity in correlation with increasing size. Why does the population of Fc receptor positive monocytes in the 24A fraction display the least ADCC activity while the essentially Fc receptor negative 28 ml/minute monocytes show moderate activity? It is known that Fc receptors are necessary for participation in ADCC. The fact that Fc receptors were detectable on the majority of monocytes in the 28 ml/minute fraction after 4 hours in culture offers an explanation for the moderate ADCC activity assayed at 16 hours. The lack of ADCC in the small monocyte population is more difficult to explain. Our experiments demonstrated that the presence of lymphocytes in sufficient numbers interfered with detection or actually blocked ADCC activity of 28 ml/minute monocytes. This observation is consistent with work reported by McCarley et al. (128) who demonstrated that ADCC activity of both subpopulations of human CCE-separated monocytes can be suppressed by the addition of either lymphocytes or cold target cells (erythrocytes). Thus, the high lymphocyte contamination in the 24A fraction and even in the Percoll purified small monocytes may be preventing ADCC. The mechanism by which ADCC is blocked by non-effector cells is unknown at this time. As to whether a highly purified small monocyte population has the ability to participate in ADCC will have

to await the development of new methods to further purify these cells.

Eremin et al. (53) reported that the low level of ADCC in guinea pig peripheral blood was attributed to Kurloff cells. However, CCE separated and Percoll-purified Kurloff cells had negligible ADCC levels. These highly purified Kurloff cells represented 72% of the total Kurloff population. Kurloff cells like monocytes displayed differences in size and therefore CCE did separate the Kurloff cells into various fractions. At this time, it is not known whether a portion of the ADCC activity encountered in the 28 ml/minute and R/O fractions is attributed to Kurloff cells. However, this possibility seems unlikely in view of the absence of Fc receptors on Kurloff cells of all fractions and the relatively low numbers of these cells present in fractions other than the 24A fraction. The Percoll purified 24B monocytes which lacked Kurloff cells had moderate ADCC activity suggesting that the ADCC activity observed in this fraction was not due to Kurloff cells. The lack of platelets in CCE-fractionated preparations as well as the inactivity of purified lymphocytes in our ADCC assay preclude these known Fc receptor bearing components as being responsible for ADCC activity in the guinea pig.

The experiments designed to evaluate tumoricidal activity suggest that the small monocyte is a candidate for the mediation of native tumoricidal activity against the

xenogeneic P-815 tumor target. This conclusion is based on the following:

1. Guinea pig mononuclear preparations demonstrate native tumoricidal activity against the xenogeneic P-815 mastocytoma at 16 hours but not at 4 hours.

Native tumoricidal activity manifested by NK cells of lymphoid origin typically takes place within 4 hours (82). Furthermore, macrophages and monocytes are known to be tumoricidal against xenogeneic carcinomas whereas NK cells are reactive against a more restricted group of hematopoietic cancers (80,82,94,241).

2. Counter-flow centrifugation elutriation isolated this native tumoricidal activity to the 24A small monocyte fraction. Little activity was found in the 28 ml/minute fraction of intermediate-sized monocytes.

3. Percoll purification of the 24A fraction showed localization of tumoricidal activity to the small monocyte-enriched fraction. Negligible activity was found in the lymphocyte or Kurloff cell fractions after Percoll enrichment. Thus, the majority of lymphocytes and Kurloff cells were not tumoricidal towards a P-815 mastocytoma target.

The above considerations argue for the small monocyte as one cell capable of mediating native tumoricidal activity in the guinea pig. A similar situation exists in

man with small monocytes possessing native tumoricidal activity against a variety of tumors and large monocytes being incapable of tumor destruction in an unstimulated state (150,151). Unfortunately, the lack of a purified population of small monocytes even after Percoll enrichment prevents a definitive identification of the cell responsible for native tumoricidal activity in the guinea pig.

Monocytes of the R/O fraction have not heretofore been described. These monocytes had the highest degree of adherence, esterase activity, phagocytosis, Fc receptor avidity and ADCC activity but the lowest amount of peroxidase reactivity of all monocyte populations studied. The R/O monocytes were rarely seen on peripheral blood smears as their concentration is estimated at 0.14% of the total white cell population. Even in the post Ficoll-Hypaque fraction they constituted only 0.7% of the total cells (7% of the total monocytes). This R/O fraction also contained myeloid precursors (promyelocytes, myelocytes and metamyelocytes) which were not observed in 500 cell differentials of peripheral blood smears. The R/O monocytes were much larger than the granulocytes and Kurloff cells contained in the same fraction. The finding of myeloid precursors and large monocytes in the R/O fraction underscores the potential of CCE to concentrate cells present in very small numbers.

### Monocyte Heterogeneity

It is our conclusion that significant physical and functional differences exist among the monocytes separated by CCE. These properties are summarized in Tables 20 & 21. We entertain three possible interpretations of these data: (1) the separate classes of monocytes represent selection by elutriation from a normal distribution of monocyte sizes and functions, (2) the various sized monocytes represent a maturation spectrum of monocytes within the peripheral blood, and (3) the different monocyte fractions represent discrete monocyte subpopulations. Each of these possibilities will now be considered.

Given the small difference in the diameter of guinea pig mononuclear cells and the resolution capabilities of the elutriator, the different monocyte fractions may represent a spectrum of monocytes artificially divided into fractions. However, this possibility seems unlikely in view of the consistent heterogeneity in physical and functional properties found between the CCE fractions.

The various sized monocytes may represent the maturation spectrum of monocytes within the peripheral blood. Increasing histochemical staining intensity, phagocytic ability, adherence properties and ADCC activity correlated with the increased size of the monocytes. This suggests that the small monocytes are the most immature while the R/O cells are the most mature. The argument rests on the belief that promonocytes just released into

the peripheral blood from the bone marrow would not be expected to possess large amounts of specific products or be fully functional. An exception is noted for native tumoricidal activity which has been attributed to promonocytes but not fully mature monocytes (119). By these criteria, the small monocytes identified in the 24 ml/minute fraction are akin to promonocytes while intermediate-sized monocytes (28 ml/minute) represent the differentiated state of guinea pig monocytes in peripheral blood. The macrophage-like properties of the R/O cells argue for this cell being well differentiated. It possesses high esterase and low peroxidase activity, strong adherence and a high phagocytic rate. That small and large monocytes possess Fc receptors while the majority of circulating monocytes (intermediate-size) lack them has similarities to the macrophage Ia antigen which is believed to be lost and then resynthesized after phagocytosis (17,210). Monocytes newly emigrated from the bone marrow (24A) may possess receptors such as Fc whose expression is diminished during transit in the circulation. Upon maturation or differentiation due to stimulation, these cells may again express receptors and acquire the typical properties ascribed to macrophages and incidentally to R/O monocytes. Indeed, this maturation/differentiation step may enable monocytes to exit the circulation and enter the tissue compartments. The rapid expression of Fc receptors

by the 28 ml/minute monocytes in culture demonstrates the rapidity by which the differentiation events can occur.

The various-sized monocytes could represent distinct subpopulations differing quantitatively in their functional properties. There are major physical and functional differences in the 24A, 28 ml/minute and R/O monocytes. This argument gains support from examining the characteristics of 24B monocytes which appear to be a mixture of small and intermediated-sized monocytes. The 24B monocytes are more adherent, phagocytic and active in ADCC than 24A monocytes but all values are less than those of 28 ml/min monocytes. Additionally, there is a moderate degree of tumoricidal activity and Fc receptor expression in 24B monocytes compared to the higher values obtained with 24A monocytes and the negligible levels of 28 ml/min monocytes. Almost all the characteristics of these monocytes suggest a mixture of small and intermediate-sized monocytes. Such a situation could arise if a small percentage of 24A monocytes were not purged in the first 200 ml collection volume and a few intermediate-sized monocytes nonspecifically exit the elutriation chamber due to limiting flow dynamics associated with the chamber itself. This is further substantiated by re-eluting the reconstituted 24A and 28 ml/min fraction which again yielded a 24B fraction, but of higher monocyte purity. This would be expected if very few intermediate-sized lymphocytes were present in the starting preparation. The

data on Fc receptors rather than representing a maturation sequence from small to large monocytes may better be explained on the basis of 2 subpopulations of monocytes, one of which possesses and the other lacks Fc receptors. The Fc receptor positive cells may possess a rather broad size distribution and comprise all the monocytes on either end of the monocyte peak but constitute only a small proportion of the monocytes at the modal volume where the Fc receptor negative population resides (28 ml/minute). This would account for the 12%-15% of intermediate-sized monocytes which are Fc receptor positive. The rapid expression of Fc receptors by 28 ml/minute monocytes in culture then may represent an induction rather than a maturation/differentiation event. As demonstrated by Walker and co-workers with Ia antigen (234), some macrophage stem cells constitutively produce positive colonies for Ia antigen whereas others do not. Upon induction by appropriate stimuli these negative macrophage colonies will become Ia positive. Thus two individual macrophage precursor cells may exist in the guinea pig giving rise to Fc receptor positive and Fc receptor negative monocytes. Some investigators have reported an Fc receptor positive and an Fc receptor negative monocyte population in man (152). However, this has not been confirmed in our laboratory as we find that all CCE-separated human monocytes possess Fc receptors (128).

Regardless of the correct interpretation of these monocyte populations their existence is a clear demonstration of monocyte heterogeneity and establishes the guinea pig as an excellent model for studying the origin of such heterogeneity.

### Synthesis

In conclusion, the specific aim of this study was achieved: namely to develop an animal model to study monocyte heterogeneity. Monocytes of the guinea pig were isolated in sufficient quantity for functional characterization by CCE separation. Furthermore, the animals did not have to be exsanguinated for this procedure and could be reused after an appropriate time interval. The isolated monocytes were viable and demonstrated functional variations.

All criteria for the isolation of monocytes and for the demonstration of monocyte heterogeneity, as set forth in the specific aims, have been fulfilled. First, all monocytes were isolated from the peripheral blood in a reproducible fashion by using high density Ficoll-Hypaque which recovered all monocytes and depleted the mononuclear cell preparation of granulocytes and erythrocytes. Second, sufficient cells were obtained after separation to functionally characterize the monocyte fractions. If necessary, more than one elutriation run could be performed to obtain the necessary amount of cells without appreciable

loss of time. Third, the separation method was reproducible with little alteration between experiments in the fractions isolated or their functionality. Lastly, no apparent alteration in physical and functional properties were attributable to the separation procedures. Although all factors cannot be excluded, CCE caused less perturbation of function than commonly used adherence techniques. Cells were obtained quickly and in high purity allowing assays to be done in suspension culture or by whatever assay method most appropriate. In conclusion, the guinea pig has proven to be a desirable model for studying monocyte heterogeneity as well as monocyte biology.

TABLE 20. PHYSICAL AND HISTOCHEMICAL PROPERTIES OF GUINEA PIG MONOCYTES ISOLATED BY COUNTERFLOW CENTRIFUGATION ELUTRIATION.

Determination	Monocytes in Elutriation Fractions			R/O
	24A	24B	28 ml/min	
Relative concentration in peripheral blood <sup>a</sup>	35%	18%	40%	7%
Purity: OCE fractions	5%	64%	81%	43%
Percoll fractions	35%	85%	85%	NT
Volume ( $\mu^3$ )	283	300	317	354
Mean Density (g/ml)	1.075	1.075	1.075	NT
Adherence	Weak	Intermediate	Moderately Strong	Strong
Non-specific esterase	1+	1+ or 2+	2+	4+
Peroxidase	41% positive	45% positive	33% positive	Negative
Acid Phosphatase	0	2+	2+	2+

<sup>a</sup>Guinea pig peripheral blood averages 7,100 white blood cells/mm<sup>3</sup> of which monocytes constitute about 2% (150 monocytes/mm<sup>3</sup>).

NT = not tested

TABLE 21. FUNCTIONAL PROPERTIES OF GUINEA PIG MONOCYTES

Determination	Monocytes in Elutriation Fractions		
	24A	24B	28 ml/min
R/O			
Phagocytosis: Antibody-independent (Latex beads)	1+	2+	2+
			4+
Antibody-dependent (IgG-SRBC)	+	2+	2+
	-		4+
C3-dependent (Opsonized <i>Candida albicans</i> )	2+	2+	3+
			4+
Fc Receptors: Native <sup>b</sup>	99%	36%	12%
			85%
48 hr culture	96%	81%	98%
ADCC	+	+	2+
	-	-	
Native tumoricidal activity <sup>c</sup>	4+	2+	-
			1+

<sup>a</sup>Guinea pig peripheral blood averages 7,100 white blood cells/mm<sup>3</sup> of which monocytes constitute about 2% (150 monocytes/mm<sup>3</sup>).

<sup>b</sup>Tested within 2 hrs of isolation.

<sup>c</sup>16 hour assay.

NT = not tested

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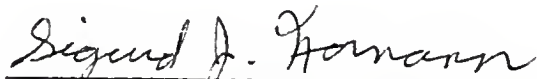
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## BIOGRAPHICAL SKETCH

Stephen Joseph Noga was born on May 27, 1954, in York, Pennsylvania. He graduated from the William Penn Senior High School in York, June, 1972. He then entered the University of Florida in September, 1972, and graduated cum laude with a B.S. in medical technology in August of 1976. He was admitted to the graduate program of the Department of Pathology at the University of Florida in September, 1978, where he has worked in the laboratory of Dr. Sigurd J. Normann. He is presently a candidate for the degree of Doctor of Philosophy with specialization in tumor biology. During his graduate career he has held a full time position as a medical technologist in the Division of Clinical Chemistry, Shands Teaching Hospital Inc., Gainesville, Florida, under the direction of Dr. Noel Maclaren. In September of 1983, Stephen will enter the M.D. program at the Johns Hopkins School of Medicine in Baltimore, Maryland. It is his intention to pursue a career in academic medicine which will integrate his interests in research, teaching and laboratory medicine.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



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Sigurd J. Normann, M.D., Ph.D., Chairman  
Professor of Pathology

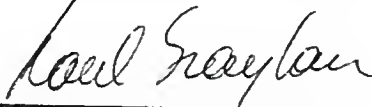
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



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Roy S. Weiner, M.D.  
Professor of Immunology and Medical  
Microbiology

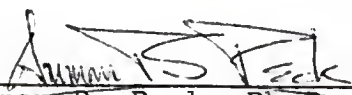
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



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Raul C. Braylan, M.D.  
Professor of Pathology

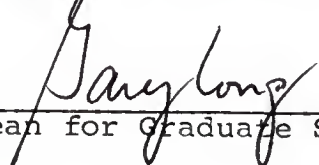
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Ammon B. Peck, Ph.D.  
Assistant Professor of Pathology

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 1983

  
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Dean, College of Medicine

  
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Dean for Graduate Studies and Research

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